[CANCER RESEARCH 63, 3247–3256, June 15, 2003]

The Different Biological Effects of Telomestatin and TMPyP4 Can Be Attributed to Their Selectivity for Interaction with Intramolecular or Intermolecular G-Quadruplex Structures

Mu-Yong Kim, Mary Gleason-Guzman, Elzbieta Izbicka, David Nishioka, and Laurence H. Hurley[1]

College of Pharmacy [M-Y. K., M. G-G., L. H. H.], and Department of Chemistry [L. H. H.], The University of Arizona, Tucson, Arizona 85721; Arizona Cancer Center, Tucson, Arizona 85724 [M-Y. K., M. G-G., L. H. H.]; Institute for Drug Development, San Antonio, Texas 78245 [E. I.]; and Department of Biology, Georgetown University, Washington, DC 20057 [D. N.]

ABSTRACT

Demonstration of the existence of G-quadruplex structures in telomeres of *Stylonychia macronuclei* and in the promoter of c-myc in human cells has validated these secondary DNA structures as potential targets for drug design. The next important issue is the selectivity of G-quadruplex-interactive agents for the different types of G-quadruplex structures. In this study, we have taken an important step in associating specific biological effects of these drugs with selective interaction with either intermolecular or intramolecular G-quadruplex structures formed in telomeres. Telomestatin is a natural product isolated from *Streptomyces anulatus* 3533-SV4 and has been shown to be a very potent telomerase inhibitor through its G-quadruplex interaction. We have demonstrated that telomestatin interacts preferentially with intramolecular *versus* intermolecular G-quadruplex structures and also has a 70-fold selectivity for intramolecular G-quadruplex structures over duplex DNA. Telomestatin is able to stabilize G-quadruplex structures that are formed from duplex human telomeric DNA as well as from single-stranded DNA. Importantly, telomestatin stabilizes these G-quadruplex structures in the absence of monovalent cations, which is a unique characteristic among G-quadruplex-interactive compounds. At noncytotoxic concentrations, telomestatin suppresses the proliferation of telomerase-positive cells within several weeks. In contrast, TMPyP4, a compound that preferentially facilitates the formation of intermolecular G-quadruplex structures, suppresses the proliferation of alternative lengthening of telomeres (ALT)-positive cells as well as telomerase-positive cells. We have also demonstrated that TMPyP4 induces anaphase bridges in sea urchin embryos, whereas telomestatin did not have this effect, leading us to conclude that the selectivity of telomestatin for intramolecular G-quadruplex structures and TMPyP4 for intermolecular G-quadruplex structures is important in mediating different biological effects: stabilization of intramolecular G-quadruplex structures produces telomerase inhibition and accelerated telomere shortening, whereas facilitation of the formation of intermolecular G-quadruplex structures induces the formation of anaphase bridges.

INTRODUCTION

Telomeres are specialized functional DNA-protein structures that consist of a long stretch of double-stranded tandem repeats, d[TTAGGG/CCCTAA]$_n$, and a short, single-stranded G-rich[2] 3′-overhang (1, 2). Griffith *et al.* (3) have shown that mammalian telomeres are arranged into large duplex loop-back structures (T-loops) *in vivo*, which are formed through the invasion of the single-stranded telomeric 3′-overhang into the duplex telomeric repeat. Their recent studies demonstrated that some portion of the cytosine-rich (C-rich) strand of the telomeric junction might also invade the duplex, resulting in the formation of a Holliday junction-like structure (4). In addition, Karlseder *et al.* (5) demonstrated that overexpression of TRF2, a telomeric DNA binding protein, protects critically short telomeres from fusion and repressed chromosome-end fusions in presenescent cultures, which explains the ability of TRF2 to delay senescence. These results suggest that the ends of telomeres are structurally more complex than generally acknowledged. In normal cells, telomeres are progressively shortened by 50–200 bases after each round of cell division because of the inability of endogenous DNA polymerase to fully replicate the lagging telomeric DNA strand (6, 7). Critically short human telomeres cannot form secondary structures and thereby induce senescence either by activating p53 or by inducing the p16/RB pathway, a process that initiates growth arrest and cell death (8, 9). Under rare circumstances a cell can escape this stage and become immortal by stabilizing the length of its telomeres, usually through the activation of the enzyme telomerase (10). Telomerase consists of two major components, a functional or template RNA (hTR) and an hTERT catalytic subunit, and is responsible for the maintenance of telomere length by adding a telomeric repeat onto the 3′-ends of chromosomes. Active telomerase has been detected in a majority of human cancer cells but not in normal somatic cells, which has made telomerase an attractive target for the design of anticancer drugs. There have been a number of reports on different strategies for inhibiting telomerase activity in human cells (11, 12).

Sequestering the substrate of telomerase, which is a single-stranded telomeric DNA, as a G-quadruplex is a reasonable approach to the inhibition of telomerase activity (13, 14). The noncoding repeat sequences of guanine/thymine-rich (GT-rich) DNA, which contain the 3′-overhang of human telomeres, have been shown to form tetrastranded DNA structures termed G-quadruplexes. Wang and Patel (15) reported that a DNA oligomer with a human telomeric sequence forms an intramolecular basket-type G-quadruplex structure (Fig. 1*A*) in the presence of sodium. More recently, Parkinson *et al.* (16) have reported an intramolecular propeller-type G-quadruplex structure (Fig. 1*A*) that is preferentially formed in the presence of a G-quadruplex-stabilizing compound and is also stabilized by potassium. Other forms of G-quadruplex structures exist *in vitro* (Fig. 1*A*) and can be classified in terms of strand stoichiometry and strand orientation (17, 18). DNA sequences containing two or more G-rich repeats have been shown to form G-G hairpins, which in turn dimerize to form several types of stable dimeric quadruplexes, and a single G-rich repeat within a DNA sequence allows the formation of intermolecular quadruplexes (Fig. 1*A*). The identification of chaperone proteins that facilitate the formation of G-quadruplexes, as well as proteins that recognize and bind to G-quadruplexes and helicases that selectively unwind G-quadruplexes, strongly support the existence of G-quadruplexes *in vivo* (19–22). Recently, the *in vivo* existence of G-quadruplexes in telomeres has been demonstrated by antibody studies in *Stylonychia macronuclei* (23).

It has been proposed that small organic molecules that stabilize or induce G-quadruplex structures are likely to inhibit telomerase activity by sequestration of the substrate required for this activity, although the biological effects of these molecules may be more directly related to telomere disruption (12–14). Indeed, a number of G-quadruplex-

Received 11/15/02; accepted 4/9/03.

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[1] To whom requests for reprints should be addressed, at Phone: (520) 626-5622; Fax: (520) 626-5623; E-mail: hurley@pharmacy.arizona.edu.

[2] The abbreviations used are: G-rich, guanine-rich; hTERT, human telomerase reverse transcriptase; DMS, dimethyl sulfate; ALT, alternative lengthening of telomeres.

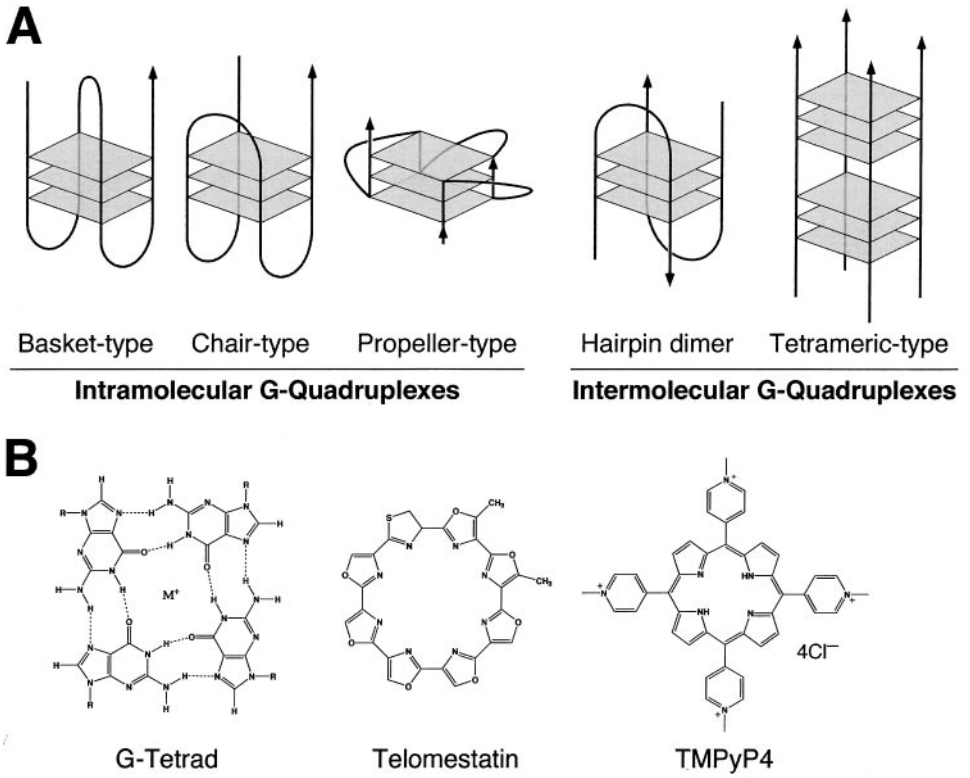

Fig. 1. *A*, various types of intra- and intermolecular G-quadruplexes. *B*, structures of a G-tetrad and the G-quadruplex-interactive compounds telomestatin and TMPyP4.

interactive compounds have been reported to inhibit telomerase activity, and some of them have shown encouraging data beyond telomerase inhibition, including telomeric disruption and short-term biological effects, such as formation of anaphase bridges, apoptosis, and *in vivo* activity in mouse xenograft models (24–26). TMPyP4 (Fig. 1*B*) has been shown to inhibit telomerase activity in MCF7 breast tumor cells (27) and stabilization of anaphase bridges (24). A 9-anilino proflavine derivative has been optimized to interact with the intramolecular G-quadruplex from human telomere and to minimize interaction with duplex DNA. This compound has 60–100 nM potency in a modified telomeric repeat amplification protocol assay (28). The triazines have been demonstrated to produce telomere shortening, which is associated with delayed growth arrest and cell senescence (29). A novel pentacyclic acridine has been shown to inhibit telomerase activity in 21NT cells, which was accompanied by an increase in cells in the G_2-M phase of the cell cycle and a lower expression of the *hTERT* gene. This compound also induced a cessation of growth of GM847 cells, which maintain telomeres by an ALT mechanism (30). The fluoroquinophenoxazines are redesigned topoisomerase II poisons that now interact more specifically with G-quadruplex structures (31). A subsequent generation of fluoroquinoanthroxazines has also been designed and synthesized to have selectivity for either topoisomerase II or G-quadruplex interactions (32).

Telomestatin (Fig. 1*B*) is a natural product isolated from *Streptomyces anulatus* 3533-SV4 and has been shown to be a very potent telomerase inhibitor (33). Significantly, telomestatin appears to be a more potent inhibitor of telomerase (5 nM) and, in comparison to TMPyP4, is at least two orders of magnitude more potent (32). The structural similarity between telomestatin and a G-tetrad suggested that the telomerase inhibition (33) might be attributable to the ability of telomestatin to interact directly with G-quadruplex structures and thereby sequester single-stranded $d[TTAGGG]_n$ primer molecules required for telomerase activity (34). Subsequently, we provided the experimental evidence that telomestatin interacts with G-quadruplex structures (34).

In this study, we demonstrate the preference of telomestatin for the intramolecular, rather than the intermolecular, G-quadruplex structure, and also its selectivity for the G-quadruplex structure over a single-stranded or duplex DNA structure. The kinetics of binding of telomestatin to the intramolecular structure, the stability of the complex, and its susceptibility to S1 nuclease are also measured. On the basis of the selectivity of telomestatin for intramolecular G-quadruplex structures and TMPyP4 for intermolecular G-quadruplex structures, cellular studies were designed to determine the corresponding biological effects.

MATERIALS AND METHODS

Materials, Enzymes, and Drugs. A stock solution of telomestatin (1 mM) was dissolved in DMSO and diluted to working concentrations with distilled water immediately before use. Acrylamide/bisacrylamide solution and ammonium persulfate were purchased from Bio-Rad and *N,N,N′,N′*-tetramethylethylenediamine was purchased from Fisher. T4 polynucleotide kinase and Taq DNA polymerase were purchased from New England Biolabs and Promega, respectively. [γ-^{32}P]ATP was purchased from NEN DuPont.

Preparation and End Labeling of Oligonucleotides. Oligonucleotides were synthesized on an Expedite 8909 nucleic acid synthesis system (PerSeptive Biosystems, Framingham, MA) using the phosphoramidite method. The oligonucleotides were eluted from the column with aqueous ammonia and deprotected by heating at 55°C overnight, followed by 15% denaturing polyacrylamide gel purification. Before the experiments, all of the oligonucleotides were treated in 10 mM NaOH at 37°C for 30 min, followed by neutralization with 10 mM HCl and ethanol precipitation to disrupt the self-associated structures. The 5′-end-labeled single-strand oligonucleotide was obtained by incubating the oligomer with T4 polynucleotide kinase and [γ-^{32}P]ATP at 37°C for 1 h. Labeled DNA was purified on a Bio-Spin 6 chromatography column (Bio-Rad) after inactivation of the kinase activity by heating at 70°C for 8 min.

Electrophoretic Mobility Shift Assay. End-labeled oligomer (5 nM) was incubated in 10 μl of buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 1.5 μg/μl BSA] at 20°C for 10 min. The incubation was continued for an additional 30 min after the addition of various concentrations of

telomestatin to the mixture. Samples were analyzed by 12% native polyacrylamide electrophoresis with 1× tris-borate-EDTA buffer as a running buffer. For the time-course experiment, the samples were taken at the times specified in the figures and loaded onto a 12% native polyacrylamide gel. For the experiment with double-stranded DNA, the labeled strand was annealed with the complementary DNA and purified on an 8% native polyacrylamide gel. The labeled double-stranded DNA was incubated in the same buffer at 37°C and 55°C for 15 h. To measure the effects of telomestatin and TMPyP4 on the formation of intermolecular G-quadruplexes, 2 μM of oligomer was used.

Competition Assay. End-labeled oligomer Hu4 (5 nM; Table 1) was incubated with 0.05 μM of telomestatin at 20°C for 30 min, as described above. The G-quadruplex–drug complex was purified from the unbound telomestatin using a Bio-Spin 6 chromatography column and incubated with various concentrations of unlabeled Hu4 for an additional 40 min at 20°C. Samples were mixed with glycerol solution (5% final) and loaded onto a 12% native polyacrylamide gel.

Methylation Protection. A methylation protection experiment was performed after incubation of oligonucleotides with telomestatin, as described above. For each incubation, 10 μl of sample were mixed with 200 μl of reaction buffer [50 mM sodium cacodylate (pH 8.0) and 1 mM EDTA] and 1 μl of 100% DMS. The reaction was stopped by adding 50 μl of DMS stop buffer [1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 100 μg/ml calf thymus DNA]. Samples were then subjected to ethanol precipitation, piperidine treatment, and 12% denaturing PAGE.

Polymerase Stop Assay. The DNA primer and templates (Table 1) were synthesized and purified as described above. Labeled DNA primer (15 nM) and templates (10 nM) were annealed in buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 1.5 μg/μl BSA] with 0.1 mM dNTP by heating to 95°C and were slowly cooled to room temperature (32, 35). Taq DNA polymerase (5 units) was added and the mixture was incubated for 20 min at 55°C. The polymerase extension was stopped by adding 2× stop buffer [10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanole, and 0.1% bromphenol blue in formamide solution] and loaded onto a 12% denaturing polyacrylamide gel.

Electrophoresis and Quantification. Electrophoresis proceeded for 4 h (350 V) for the native gel and 2 h (1900 V) for the denaturing gel. The dried gels were exposed on a phosphor screen. Imaging and quantification were performed using a PhosphorImager (Storm 820) and ImageQuant 5.1 software from Molecular Dynamics.

Short-Term Cytotoxicity Assay. SW39 (telomerase-positive/ALT-negative) and SW26 (telomerase-negative/ALT-positive) were generously supplied by Dr. Jerry W. Shay (University of Texas, Southwestern Medical Center, Dallas, TX). Briefly, IMR90 cells were immortalized by SV40 T-antigen oncoprotein and separated into two subtypes: telomerase-positive/ALT-negative (SW39) and telomerase-negative/ALT-positive (SW26). Exponentially growing cells (~1–2 × 10^3 cells) in 0.1 ml of medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1-ml aliquots of medium containing graded concentrations of telomestatin and TMPyP4 were added to the cell plates. On day 4, the cell cultures were incubated with 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (1 mg/ml in Dulbecco's PBS) for 4 h at 37°C. The resulting formazan precipitate was solubilized with 200 μl of 40 mM HCl in isopropyl alcohol. For determination of the IC_{50} values, the absorbance readings at 570 nm were fitted to the four-parameter logistic equation.

Long-Term Cytotoxicity Assay. Cultures were maintained at 37°C, 5% CO_2, in a 4:1 mixture of DMEM and medium 199 (CellGro), supplemented with 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (Omega Scientific). SW26 and SW39 cells were seeded at 4 × 10^5 and 2 × 10^5 cells/75 cm^2 flask, respectively. Cells were passaged every 7 days, counted by hemocytometer, and reseeded at the original concentrations.

Anaphase Bridge Study. *Lytechinus pictus* sea urchins (Marinus Inc., Long Beach, CA) were maintained at 15°C in refrigerated aquaria containing Instant Ocean artificial seawater. Spawning, fertilization, drug treatment, and embryo processing were done as described previously (24). Briefly, 10 min after insemination, the fertilized eggs were allowed to settle, and the supernatant was aspirated and replaced with fresh artificial seawater. The embryos were cultured at 18°C. Twenty min after fertilization, the agents were added to 1% embryo suspensions. Ten h after insemination, the embryos were pelleted by centrifugation. The nuclei were stained by the Feulgen reaction, and the chromatin was visualized and photographed with an Olympus BH2 photomicroscope equipped with fluorescence optics.

S1 Nuclease Digestion. End-labeled oligomer Hu4 (5 nM) was incubated with various concentrations of telomestatin and TMPyP4 and then digested with S1 nuclease (4 units) for 20 min at 25°C in the reaction buffer [50 mM sodium acetate (pH 4.5), 280 mM NaCl, 4.5 mM $ZnSO_4$, 0.5 mM DTT, 0.1 mM EDTA, and 1.5 μg/μl BSA)]. After phenol/chloroform extraction and ethanol precipitation, samples were dissolved in loading buffer (10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanole, and 0.1% bromphenol blue in formamide solution) and loaded onto a 12% denaturing polyacrylamide gel.

Table 1 *Oligonucleotides used in this study*

Designation	Sequence
Hu4	5′-$[TTAGGG]_4$-3′
Hu-Mut	5′-$[TTAGAG]_4$-3′
26G3	5′-CCACTTTTTAAAAGAAAAGGGACTGG-3′
27G4	5′-CCACTTTTTAAAAGAAAAGGGGACTGG-3′
28G5	5′-CCACTTTTTAAAAGAAAAGGGGGACTGG-3′
29G6	5′-CCACTTTTTAAAAGAAAAGGGGGGACTGG-3′
29G6-Mut	5′-CCACTTTTTAAAAGAAAAGGGGGGTTTGG-3′
Hu6	5′-$[TTAGGG]_6$-3′
Primer	5′-TAATACGACTCACTATAG-3′
Temp [TTAGGG]	5′-TCCAACTATGTATAC$[TTAGGG]_4$TTAGCCACGCAAT-TGCTATAGTGAGTCGTATTA-3′
Temp [TTAGAG]	5′-TCCAACTATGTATAC$[TTAGAG]_4$TTAGCCACGCAAT-TGCTATAGTGAGTCGTATTA-3′

RESULTS

Telomestatin Is Much More Efficient Than TMPyP4 in Facilitating the Formation of Intramolecular G-Quadruplex Structures. The role of telomestatin in the formation of intramolecular G-quadruplex structures was investigated using an electrophoretic mobility shift assay. DNA oligomers with telomeric sequences form intramolecular G-quadruplex structures that migrate faster than nonstructured single-stranded DNA (36, 37). Oligomer Hu4, which contains four repeats of the human telomeric sequence d$[TTAGGG]_4$, was incubated with increasing concentrations of telomestatin, at 20°C for 30 min in the absence of both Na^+ and K^+. At 10-nM concentrations of telomestatin, a new high-mobility band appeared and its intensity increased in a dose-dependent manner (Fig. 2*A*, *Lanes 3–5*). The EC_{50} value, which indicates the concentration of telomestatin required to achieve 50% conversion of linear DNA to the high-mobility complex, was found to be 0.03 μM. In a parallel experiment with a mutated oligomer (Hu-Mut; see Table 1) that contains four repeats of TTAGAG instead of TTAGGG, conversion of linear DNA to the high-mobility complex formed by telomestatin was not found (Fig. 2*A*, *Lanes 6–10*), indicating that the contiguous guanine stretches play a key role in the formation of the high-mobility complex.

To further probe the structure of the high-mobility complex, a DMS protection experiment was carried out. N7 of guanine is critical for the formation of a Hoogsteen hydrogen bond with the hydrogen atom at N2 of another guanine in a G-quartet (Fig. 1*B*). Therefore, the formation of G-quadruplex structures should effectively protect N7 guanines against methylation by DMS. As shown in *Lane 3* of Fig. 2*B*, all of the guanines in oligomer Hu4 were protected from methylation in the presence of telomestatin. This DMS protection pattern is typical of G-quadruplex structures in which all of the guanines participate in G-quadruplex formation. In contrast, the protection of guanines was not detected in a control group in which the mutated oligomer was incubated with DMS in the presence of telomestatin (Fig. 2*B*, *Lanes 4–6*).

A comparison of the activity of telomestatin with TMPyP4, another G-quadruplex-interactive compound, was made under the same experimental conditions. For TMPyP4, conversion of oligomer Hu4 to an intramolecular G-quadruplex structure was not detected, even at a concentration of 20 μM (Fig. 2*C*). Thus, telomestatin is much more efficient than TMPyP4 in converting the single-stranded Hu4 into an intramolecular G-quadruplex structure. The altered mobility of the

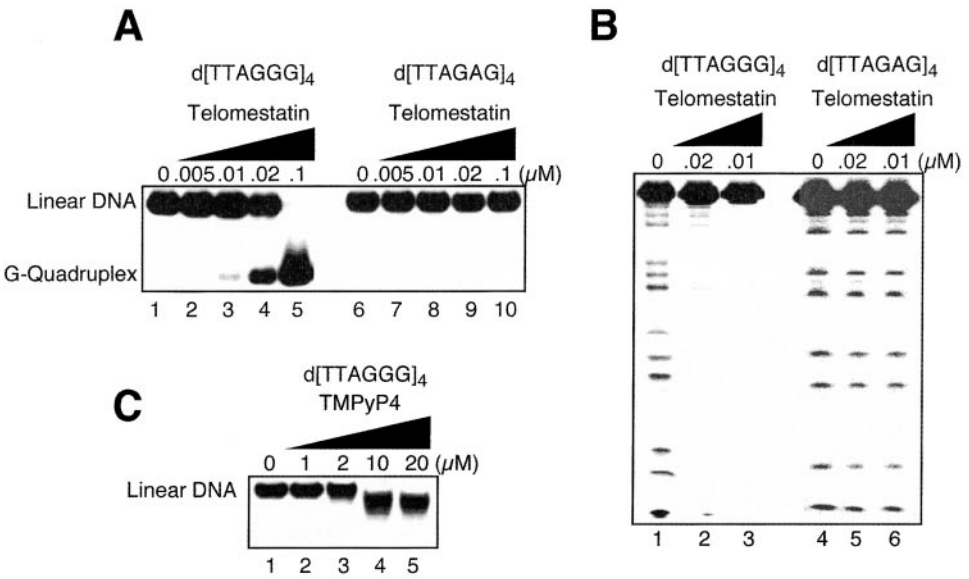

Fig. 2. Effects of telomestatin and TMPyP4 on the formation of intramolecular G-quadruplex from the human telomeric sequence d[TTAGGG]$_4$ and its mutant sequence d[TTAGAG]$_4$. *A*, end-labeled oligonucleotides were incubated for 30 min with various concentrations of telomestatin in reaction buffer. Two bands corresponding to linear DNA and G-quadruplex were identified. *B*, a methylation protection experiment was performed after incubation of oligonucleotides with telomestatin. *C*, the end-labeled oligonucleotide d[TTAGGG]$_4$ was incubated with TMPyP4 as described in *A*.

linear DNA in Fig. 2*C* is attributable to its association with the cationic porphyrin.

TMPyP4 Is More Efficient Than Telomestatin in Facilitating the Formation of Intermolecular G-Quadruplex Structures. To compare the effects of telomestatin and TMPyP4 on the formation of intermolecular G-quadruplex structures, a 29-mer oligonucleotide containing six consecutive guanines (29G6; see Table 1), which have been demonstrated previously to form interstrand G-quadruplexes (38), was incubated with increasing concentrations of telomestatin. For telomestatin, the intensity of two low-mobility complexes increased in a dose-dependent manner, with the faster complex appearing only when the telomestatin concentration was 10 μM or greater (Fig. 3*A*, *Lanes 5–7*). Under the same experimental conditions, TMPyP4 was found to increase the formation of a low-mobility complex at a concentration of 0.01 μM or greater (Fig. 3*B*, *Lanes 3–7*). These results demonstrate that TMPyP4 is more efficient than telomestatin at facilitating the formation of intermolecular G-quadruplex structures.

To determine the structures of the various complexes formed in the presence of telomestatin and TMPyP4, a mobility assay was carried out with several modified oligomers containing various numbers of guanine bases. The oligomer with five consecutive guanines (28G5; see Table 1) showed a pattern of complex formation similar to that for 29G6, whereas the oligomers with three and four consecutive guanines (26G3 and 27G4, respectively; see Table 1) could not form either of the two low-mobility complexes (Fig. 3*C*). Because 26G3 and 27G4 have the same DNA sequence as 29G6, except for the number of guanines, the low-mobility complexes formed in the presence of telomestatin (Fig. 3*A*) were a result of the specific interaction of telomestatin with the DNA structures in which guanines play important roles and most likely correspond to the intermolecular four-stranded G-quadruplex structures. The low-mobility complexes formed in the presence of TMPyP4, corresponding to the bands between the linear DNA and antiparallel G-quadruplex bands, are most likely hairpin dimers (Fig. 3*B*, *Lanes 5–7*; Ref. 39). The complex that shows the lowest mobility in the presence of TMPyP4 is most likely a DNA aggregate formed from the binding of several G-quadruplexes to each other by shared G-tetrads (Fig. 3*B*, *Lanes 4–7*).

To further characterize these intermolecular G-quadruplex structures, the two flanking bases on the 3′-side of the six consecutive guanines were mutated from the wild-type AC motif to a TT motif (*i.e.*, 29G6-Mut; see Table 1). Under these experimental conditions, 29G6 can form both parallel and antiparallel G-quadruplex structures. However, for 29G6-Mut, the antiparallel alignment of the intermolecular G-quadruplex is more thermodynamically favored than the parallel alignment, because of the four additional Watson-Crick base-pairings on both sides of the guanine tract (Fig. 3*D*). Correspondingly, we observed that 29G6-Mut formed one G-quadruplex structure, even in the absence of salt and telomestatin (Fig. 3*E*, *Lane 2*). Hence we have identified this slower mobility band as the antiparallel intermolecular G-quadruplex and the faster band of the two low-mobility complexes as the parallel intermolecular G-quadruplex.

A comparison of the formation of intramolecular (Fig. 2*A*) and intermolecular (Fig. 3*A*) G-quadruplex structures shows that telomestatin is more efficient at converting linear DNA to the intramolecular rather than the intermolecular species. However, the two oligomers that were used to compare the preference of telomestatin for intramolecular or intermolecular G-quadruplexes have different DNA sequences. Thus, a more direct comparison was made in an experiment using oligomer Hu6 (Table 1) containing six repeats of the human telomeric sequence, which can form both intramolecular and intermolecular G-quadruplexes. At higher concentrations of telomestatin, the intensity of new high-mobility bands, which correspond to intramolecular G-quadruplexes, was significantly increased, whereas there were no bands observed that would correspond to intermolecular G-quadruplex structures (Fig. 3*F*). This result provides additional evidence that telomestatin interacts preferentially with intramolecular G-quadruplexes over intermolecular G-quadruplexes. TMPyP4 does not induce either of the intramolecular or intermolecular G-quadruplexes from this sequence (data not shown).

Telomestatin Can Replace the Need for Sodium or Potassium to Stabilize Intramolecular G-Quadruplex Structures. Monovalent cations, notably sodium and potassium, have been shown to stabilize human telomeric G-quadruplex structures, presumably by coordinating with the eight carbonyl oxygen atoms present between stacked tetrads (37). To determine the importance of monovalent cations for the formation of G-quadruplex structures by telomestatin, the oligomer Hu4 was incubated with increasing concentrations of telomestatin in the presence and absence of sodium and potassium. Samples were run on a native polyacrylamide gel with 1× tris-borate-EDTA buffer without the addition of salt. Sodium and potassium ions were both found to act in synergy with telomestatin to stabilize the forma-

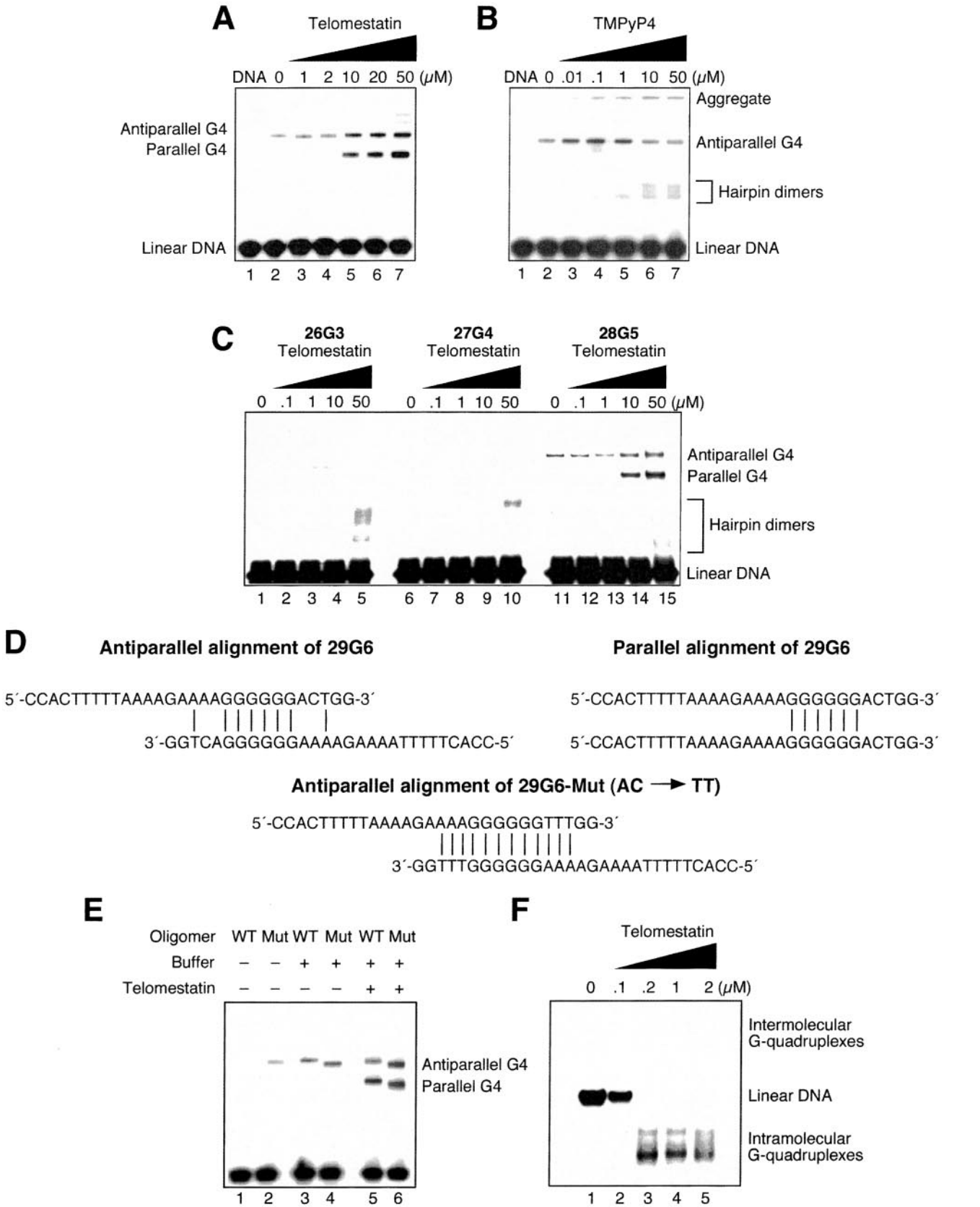

Fig. 3. Effects of telomestatin and TMPyP4 on the formation of intermolecular G-quadruplexes. *A*, the end-labeled oligonucleotide 29G6 was incubated for 2 h with various concentrations of telomestatin. Two bands corresponding to antiparallel and parallel G-quadruplexes were identified. *B*, the end-labeled oligonucleotide 29G6 was incubated with various concentrations of TMPyP4. *C*, the end-labeled oligonucleotides 26G3, 27G4, and 28G5 were incubated with increasing concentrations of telomestatin. *D*, schematic representation of the oligonucleotides and probable alignments. *E*, the end-labeled oligonucleotides 29G6 (*WT*) and 29G6-Mut (*Mut*) were incubated with increasing concentrations of telomestatin. 29G6 was used for the reaction of *Lanes 1*, *3*, and *5*, and 29G6-Mut was used for the reaction of *Lanes 2*, *4*, and *6*. *Lanes 1* and *2* were incubated in water. *Lanes 3* and *4* were incubated in the reaction buffer. *Lanes 5* and *6* were incubated with 50 μM telomestatin in the reaction buffer. *F*, the end-labeled oligonucleotide Hu6 was incubated for 2 h with increasing concentrations of telomestatin.

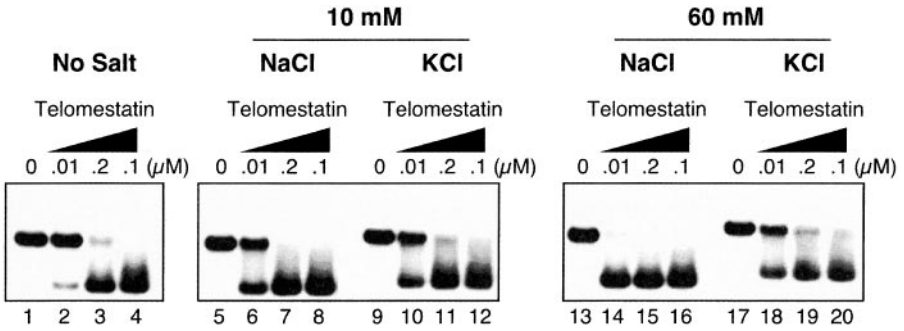

Fig. 4. Effects of telomestatin on the formation of intramolecular G-quadruplexes in NaCl and KCl. The end-labeled oligonucleotide Hu4 was incubated for 30 min with increasing concentrations of telomestatin in the absence and presence of NaCl and KCl. *Lanes 1–4* contain no monovalent cations, *Lanes 5–8* contain 10 mM NaCl; *Lanes 9–12* contain 10 mM KCl; *Lanes 13–16* contain 60 mM NaCl; *Lanes 17–20* contain 60 mM KCl.

tion of intramolecular G-quadruplex structures, and the effect of sodium was slightly stronger than that of potassium, most notably at a concentration of 60 mM (Fig. 4). EC_{50} values for the formation of the G-quadruplex structure were found to be 0.015 μM (no salt), 0.011 μM and 0.012 μM (10 mM NaCl and KCl, respectively), and $<$0.010 μM and 0.012 μM (60 mM NaCl and KCl, respectively). It is important to note that telomestatin is apparently able to convert linear DNA into a G-quadruplex structure, even in the absence of monovalent cations. This demonstrates that telomestatin can replace the need for the monovalent cations in facilitating the formation of intermolecular G-quadruplex structures. This is a unique property among G-quadruplex-interactive compounds examined to date.

Telomestatin Binds Strongly to G-Quadruplex Structures and Is Not Easily Dissociated from the G-Quadruplex–Drug Complex. A time-course experiment was used to determine the kinetics of formation of the telomestatin–G-quadruplex complex. The incubation of oligomer Hu4 with 0.1 μM telomestatin was stopped at various times, and aliquots were loaded onto a native polyacrylamide gel. It was found that 69% of the linear DNA was converted into G-quadruplex structures in the first minute, after which the rate of G-quadruplex formation remained constant, although markedly lower relative to the apparent initial rate (Fig. 5, *A* and *B*). The apparent rate of conversion in the presence of 0.1 μM telomestatin, which was derived by plotting the linear DNA concentration (*C*) to total DNA (*Co*) concentration (*C/Co*) *versus* time, was found to be 0.69 min^{-1} for the first min and 0.0046 min^{-1} thereafter, *i.e.*, a difference of 150-fold (Fig. 5*B*). One possible explanation for this dramatic rate difference is that telomestatin traps out the preformed G-quadruplex structure in the first phase that is not normally stable enough to survive during subsequent electrophoresis. In the second phase, telomestatin then binds to the newly available G-quadruplex structures as the remaining linear DNA converts to these structures. Thus, we propose that the fast reaction represents telomestatin binding to preformed G-quadruplex structures that exist at equilibrium, and the slow reaction represents the real rate of conversion of linear DNA to intramolecular G-quadruplex structures.

To determine the stability of the telomestatin–G-quadruplex complex, a competition assay was performed. The preformed telomestatin–G-quadruplex complex was incubated with increasing concentrations of cold-competitor Hu4 oligomer. If telomestatin is reversibly bound to the G-quadruplex DNA, the telomestatin that dissociates from the labeled oligomer can bind to either the labeled or unlabeled oligomer. Thus, as the relative concentration of the unlabeled oligomer increases, the chance that the labeled oligomer is replaced with unlabeled DNA in the bound complex increases. The results show that, even in the presence of a 20-fold excess of cold-competitor DNA, no loss of the preformed complex was observed (Fig. 5*C*, *Lane 7*). Therefore, telomestatin binds to the G-quadruplex structure very tightly and is not easily dissociated from it. In contrast, in a control experiment in which an excess of cold-competitor oligomer was preincubated with labeled oligomer and unbound telomestatin, the cold competitor oligomers were able to compete with the labeled DNA for telomestatin (Fig. 5*D*, *Lanes 5* and *6*).

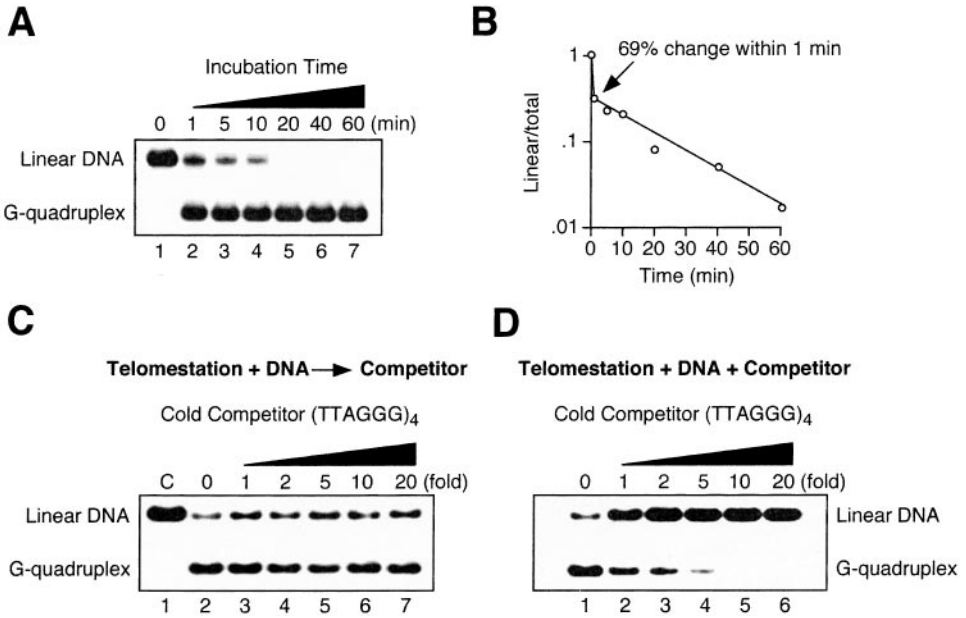

Fig. 5. Kinetics of telomestatin-assisted G-quadruplex formation. *A*, time course of G-quadruplex formation. The end-labeled oligonucleotide Hu4 was incubated with 0.1 μM telomestatin. The reaction was stopped at various time points and loaded onto a native polyacrylamide gel. *B*, graphical representation of the quantification of the gel in *A*, showing the ratio of the linear DNA against the total intensity/lane. *C*, competition assay. The end-labeled oligomer Hu4 was incubated with 0.05 μM telomestatin at 20°C for 30 min. The G-quadruplex–drug complex was purified from the unbound telomestatin using a Bio-spin 6 chromatography column and was incubated with various concentrations of unlabeled oligomer for an additional 40 min at 20°C. Samples were mixed with glycerol solution and loaded onto a native polyacrylamide gel. *D*, competition assay. The end-labeled oligomer Hu4 and various concentrations of unlabeled oligomer Hu4 were incubated with 0.05 μM telomestatin.

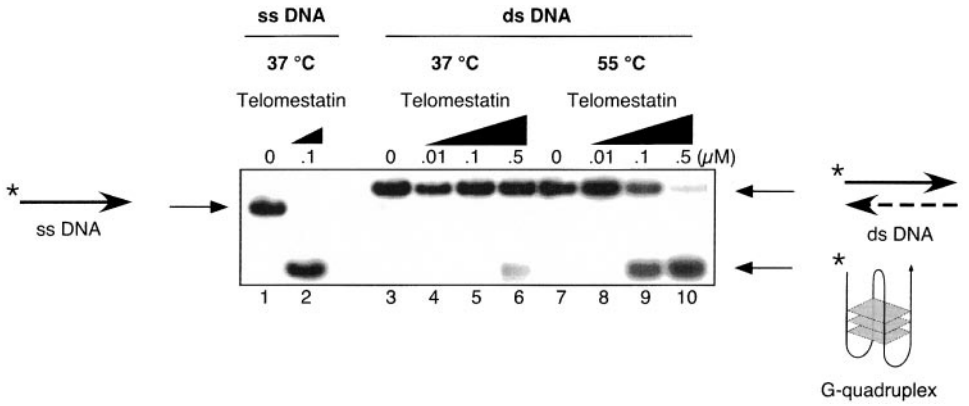

Fig. 6. Effects of telomestatin on the conversion of telomeric duplex DNA into a G-quadruplex structure. The end-labeled oligomer Hu4 was incubated at 37°C for 15 h *(Lanes 1* and *2*). Watson-Crick telomeric duplex DNA, d[TTAGGG/CCCTAA]$_4$, was incubated with increasing concentrations of telomestatin at 37°C *(Lanes 3–6)* and 55°C (*Lanes 7–10*) for 15 h. The G-rich strand was 5′-end-labeled (∗).

Telomestatin Is Able to Trap Out an Intramolecular G-Quadruplex Structure from Duplex DNA. The potential role of telomestatin in facilitating the formation of G-quadruplex structures from Watson-Crick base-paired telomeric duplex DNA was also examined. A double-stranded DNA fragment that consists of oligomer Hu4 and its complementary strand was incubated with increasing concentrations of telomestatin at 37°C for 15 h. In this experiment, the 5′-end of the G-rich strand of the duplex DNA was radiolabeled. At the highest concentration of telomestatin, a small amount of high-mobility complex, which corresponds to the intramolecular G-quadruplex structure, was observed (Fig. 6, *Lane 6*).

The conversion of duplex DNA into a G-quadruplex structure by telomestatin may be thermodynamically more favorable in chromosomal DNA because the energy for strand separation can originate from the free energy (ΔG) inherent in negative DNA supercoiling. To artificially mimic this situation in the reaction, duplex DNA was incubated at an elevated temperature (55°C) with increasing concentrations of telomestatin. Under these conditions, the amount of the intramolecular G-quadruplex bands was significantly increased, and almost all of the DNA molecules were converted to intramolecular G-quadruplex structures in the presence of 0.5 μM telomestatin after 15 h (Fig. 6, *Lane 10*).

Telomestatin Has a 70-Fold Selectivity for a G-Quadruplex Structure over Duplex DNA. In the previous sections, we have shown that telomestatin binds to intramolecular G-quadruplexes quite specifically and with high affinity. The specificity of telomestatin binding to intramolecular G-quadruplex structures *versus* single-stranded or duplex structures was investigated using a polymerase stop assay (35). A 72-mer DNA template (Temp[TTAGGG]; see Table 1) containing four repeats of the human telomeric sequence was incubated with a primer that has a complementary sequence to the 3′-end of the 72-mer template and increasing concentrations of telomestatin in the presence of Taq DNA polymerase. The principle of the assay is shown to the *left of the gel* in Fig. 7*A*. The amount of polymerase pausing at the G-quadruplex site is a direct measure of the degree of stabilization by telomestatin of the intramolecular G-quadruplex structures (35). In the absence of telomestatin there is only a slight pausing of Taq polymerization at the G-quadruplex-forming site, whereas significantly greater pausing is observed at the same position in the presence of increasing concentrations of telomestatin. At a concentration of 0.074 μM, telomestatin was found to inhibit 50% of the DNA synthesis by Taq polymerase at the G-quadruplex-forming site (Fig. 7, *A* and *B*). In a parallel experiment with a mutated template DNA that contains four repeats of TTAGAG (Temp[TTAGAG]; see Table 1), which cannot form G-quadruplex structures, there was no increase in pausing, even in the presence of high concentrations of telomestatin (Fig. 7*A*). Thus, the inhibition of polymerase activity is not attributable to the inhibition of its catalytic activity by direct interaction of telomestatin with the enzyme, but presumably is caused by the inhibition of Taq polymerase processivity by telomestatin interaction with the intramolecular G-quadruplex in the template DNA. The inhibition of polymerase processivity at the primer position in the presence of 10 μM telomestatin is most likely attributable to the affinity of telomestatin for single- and/or double-

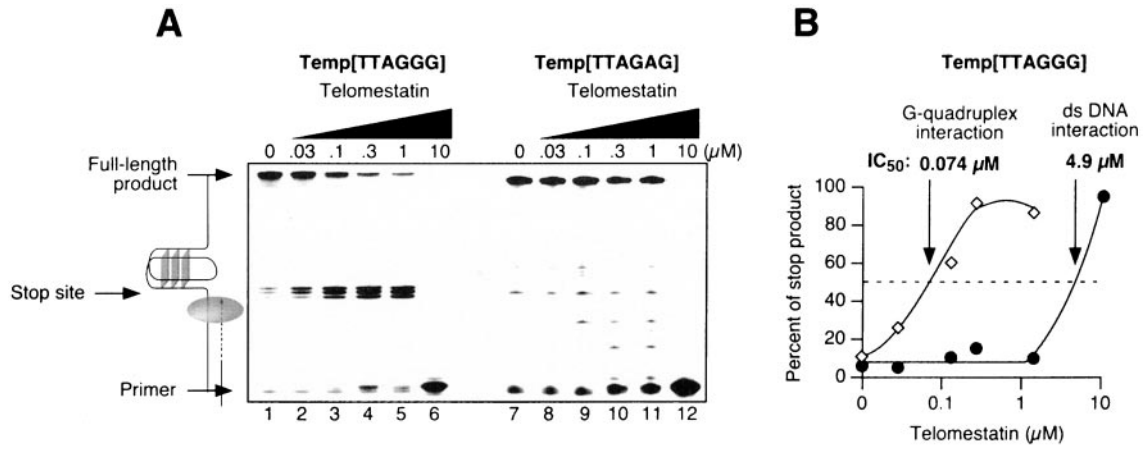

Fig. 7. *A*, concentration-dependent block of polymerase DNA synthesis by telomestatin of the G-quadruplex-stabilized structure formed on the DNA template containing the human telomeric sequence (Temp[TTAGGG] or the DNA template containing the mutant sequence (Temp[TTAGAG]. *Lanes 1–6* contain Temp[TTAGGG] and *Lanes 7–12* contain Temp[TTAGAG]. *Arrows*, the positions of the full-length product of DNA synthesis, the G-quadruplex stop site, and the free primer. *B*, graphical representation of the quantification of the *left-hand panel of the gel in A*, showing the percentage of the G-quadruplex stop product *versus* the total intensity/lane.

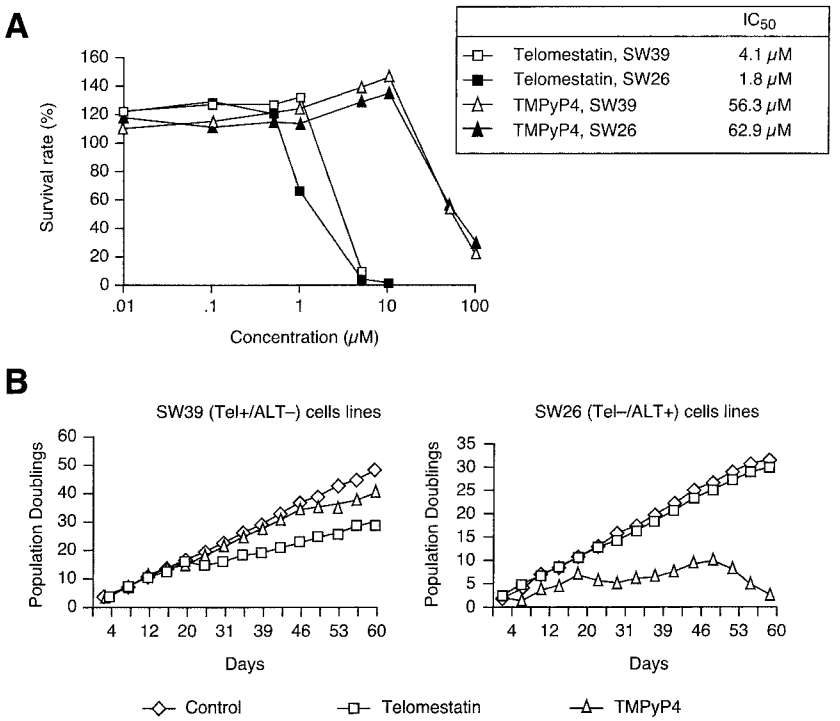

Fig. 8. Effects of telomestatin and TMPyP4 on the growth of SW39 (telomerase-positive/ALT-negative) and SW26 (telomerase-negative/ALT-positive) cell lines. *A*, short-term cytotoxicity. Cells were exposed to the indicated concentrations of compounds. Three days later the cytotoxicity was assessed and expressed as a percentage of the survivals of untreated cells (100%). Each experiment was performed four times at each point. *B*, long-term exposure with nontoxic concentrations. SW39 cells were exposed to 0.5-μM or 1-μM concentrations of telomestatin or TMPyP4, respectively. SW26 cells were exposed to 0.15-μM or 1-μM concentrations of telomestatin or TMPyP4, respectively. Each experiment was performed four times at each point.

stranded DNA. These results demonstrate that telomestatin has a high selectivity (about 70-fold) for G-quadruplex structures over single- and/or double-stranded DNA (Fig. 7*B*).

Telomestatin Suppresses the Proliferation of Telomerase-Positive Cells at Noncytotoxic Concentrations, Whereas TMPyP4 Suppresses the Proliferation of ALT-Positive Cells. In the previous sections, we have shown that telomestatin interacts preferentially with intramolecular G-quadruplexes, whereas TMPyP4 interacts with intermolecular G-quadruplex structures. To investigate the relative importance of these two different types of G-quadruplex interactions in producing the overall biological activity, the cytotoxicities of telomestatin and TMPyP4 were determined against telomerase-transformed (SW39) and ALT-transformed (SW26) cell lines, respectively. These cells maintain their telomeres either through the telomerase (telomerase-positive) and alternative lengthening of telomeres (ALT-positive) mechanisms (40, 41). As shown in Fig. 8*A*, IC_{50} values were found to be 4.1 μM (telomestatin against SW39), 1.8 μM (telomestatin against SW26), 56.3 μM (TMPyP4 against SW39), and 62.9 μM (TMPyP4 against SW26).

To further characterize the role of the two different types of G-quadruplex interactions, the long-term cytotoxic effects were compared for untreated cells and cells that had been treated for 8 weeks with noncytotoxic concentrations of telomestatin (SW39, 0.5 μM; SW26, 0.15 μM) and TMPyP4 (1 μM for both cell lines). In SW39 (telomerase-positive/ALT-negative) cells, we observed the suppression of cell proliferation within 3 weeks with telomestatin, whereas cells treated with TMPyP4 showed the suppression of cell proliferation only after 6 weeks of treatment (Fig. 8*B*). In SW26 (telomerase-negative/ALT-positive) cells, TMPyP4 induced the suppression of cell proliferation after 2 weeks, whereas the presence of telomestatin did not affect the growth curve relative to that of the control cells.

TMPyP4 Induces Anaphase Bridges in Sea Urchin Embryos, Whereas Telomestatin Does Not Have This Effect. Chromosome-specific effects of telomestatin and TMPyP4 were determined in sea urchin embryos using high-power fluorescence microscopy. During anaphase, the chromosomes of cells separate and move to opposite poles clearly with very little "tailing" of chromosome arms on the mitotic spindle. Telophase chromosomes are observed as two small, dense concentrations of mitotic chromosomes at opposite poles, at which they will condense into interphase nuclei. The control group and the samples treated with 2.5 μM of telomestatin show the normal, tight mitotic chromosomes among a large number of nuclei.[3] On the other hand, in the TMPyP4-treated (10 μM) embryos, the mitotic chromosomes are more diffuse and segregate abnormally, and end-to-end fusions are often observed. (A more complete description of these experiments with TMPyP4 and TMPyP2 is given in Ref. 24.)

Telomestatin but Not TMPyP4 Increases DNA Cleavage by S1 Nuclease at Both Loop Regions in an Asymmetric Way in a G-Quadruplex Structure. S1 nuclease degrades single-stranded DNA and RNA endonucleolytically to yield 5′-phosphoryl-terminated products, and it also cleaves double-stranded nucleic acids at nicks and small gaps. This enzyme reacts less efficiently with double-stranded DNA, double-stranded RNA, DNA/RNA hybrids, and secondary DNA structures (42, 43). The effect of telomestatin and TMPyP4 on the S1 endonuclease cleavage activity of the drug-modified G-quadruplex structure was investigated. After incubation with two different concentrations of telomestatin, the oligomer Hu4 was exposed to S1 nuclease for 10 min at 25°C. At higher concentrations of telomestatin, the intensity of S1 nuclease-mediated DNA cleavage increased significantly, especially at the DNA sequences that correspond to the two loop regions of the intramolecular G-quadruplex, whereas no apparent increase of DNA cleavage was detected with TMPyP4 (Fig. 9). Significantly, the cleavage was asymmetric, suggesting that the G-quadruplex structure and its telomestatin-modified form were also asymmetric, *i.e.*, the basket form rather than the propeller form.

[3] E. Izbicka and D. Nishioka, unpublished observations.

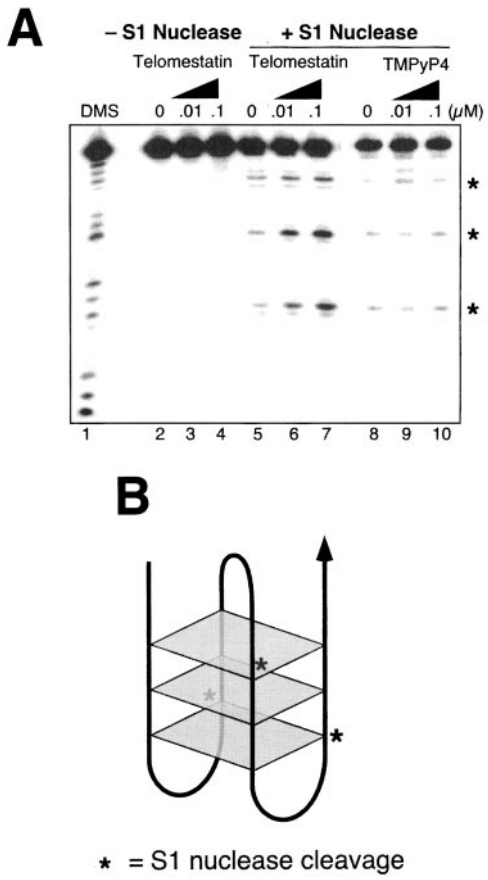

Fig. 9. Effect of telomestatin and TMPyP4 on the S1 nuclease cleavage of the human telomeric G-quadruplex. *A*, the end-labeled oligomer Hu4 that was preincubated with different concentrations of telomestatin or TMPyP4 was digested with S1 nuclease in the reaction buffer, as described in the experimental section. After phenol/chloroform extraction and ethanol precipitation, samples were loaded onto a 12% denaturing gel. *Lane 1*, DMS sequencing for guanine. *Lanes 2–4* were incubated with increasing concentrations of telomestatin in the absence of S1 nuclease. *Lanes 5–7* and *8–10* were incubated with increasing concentrations of telomestatin and TMPyP4, respectively, in the presence of S1 nuclease. *B*, *, the positions of increased DNA cleavage by S1 nuclease in the intramolecular basket-type G-quadruplex structure.

DISCUSSION

DNA is most often regarded as a duplex structure in which two self-complementary strands are held together by Watson-Crick base pairing. However, certain DNA sequences can form unique secondary DNA structures. Most notably, simple repetitive DNA sequences with a G-rich composition can readily form G-quadruplex structures under physiological conditions *in vitro*. It has been suggested that G-quadruplex structures are involved in many cellular events, such as chromosomal alignment, replication, and recombination (14). They may also act to regulate important genes, such as the oncogene *c-myc*. Recently, we have demonstrated that a specific G-quadruplex structure formed in the c-*MYC* promoter region functions as a transcriptional repressor element (44). Furthermore, we established the principle that c-*MYC* transcription can be controlled by ligand-mediated G-quadruplex stabilization (44). G-quadruplex-forming sequences are also found in several other regulatory regions of important oncogenes, including c-*MYB*, c-*FOS*, and c-*ABL*, suggesting that G-quadruplex structures might more generally play important roles in transcriptional regulation (45). The facile interconversion, under physiological conditions, between double- or single-stranded DNA and G-quadruplex structures, together with their unique structural features, makes these G-quadruplex structures attractive targets for anticancer drug design (14, 45).

It is known that the formation of most G-quadruplex structures is a slow process that takes at least several hours in the presence of high concentrations of monovalent cations (39, 46), although there are exceptions (44). However, telomestatin is able to facilitate the formation of and/or stabilize preformed intramolecular G-quadruplex structures within one minute, even in the absence of monovalent cations. Moreover, once telomestatin binds to intramolecular G-quadruplex structures, it is not easily displaced. In contrast to other G-quadruplex-interactive compounds, such as TMPyP4, telomestatin is more selective and more tightly bound to intramolecular G-quadruplexes. Previously, Shin-ya *et al.* (33) reported that telomestatin is a potent telomerase inhibitor. They also reported that telomestatin accelerates the rate of telomere shortening to a greater extent than was expected by the number of population doublings alone and that this is accompanied by cell growth arrest and senescence-associated morphological changes (47). Of the drugs evaluated in the present study (*i.e.*, TMPyP4, TMPyP2, and telomestatin), only telomestatin produced a significant decrease in telomere length (~1 kb after 39 days) in the SW26 cell line.[4] It is presumably the formation and stabilization of intramolecular G-quadruplex structures from single-stranded telomeric DNA in the presence of telomestatin that results in telomerase inhibition, because of the sequestration of single-stranded $d[TTAGGG]_n$ primer molecules in a similar way to K^+ (48). Telomestatin also increases DNA cleavage by S1 nuclease at the loop regions of intramolecular G-quadruplex structures formed with human telomeric sequences. Thus, the effect of telomestatin on the activities of both telomerase and S1 nuclease and similar DNA nucleases may play a key role in accelerated telomere shortening in cancer cells. In this study, we have demonstrated, using a polymerase stop assay, that the specific binding of telomestatin with intramolecular G-quadruplex structures causes the inhibition of DNA polymerase processivity at the human telomeric sequence, which might be an additional mechanism for accelerated telomere shortening by telomestatin.

It has been demonstrated that telomeric function is more likely to depend on structure, rather than on length alone (5). The maintenance of normal telosome structure is important for cell survival; consequently, the loss of normal telomere capping leads to apoptosis and cell death (3, 8). The selective interaction of telomestatin with intramolecular G-quadruplex structures would also be anticipated to have an influence on telomeric structure. For example, sequestration of the single-stranded 3′-overhangs of telomeres as an intramolecular G-quadruplex structure would prevent the formation of appropriate telomeric structures, such as T-loops.

Telomestatin suppresses the proliferation of telomerase-positive cells at noncytotoxic concentrations. Unlike telomestatin, TMPyP4 suppresses the proliferation of ALT-positive cells as well as telomerase-positive cells within several weeks at noncytotoxic concentrations. The unique activity of TMPyP4 against ALT-positive cells is most likely attributable to its ability to facilitate the formation of and stabilize these structures formed from adjacent telomeric ends of sister chromatids. Accordingly, in sea urchin embryo cells, TMPyP4 induces the end-to-end adherence of anaphase and telophase chromosomes, whereas telomestatin lacks this effect. This difference can, therefore, be rationalized as the selectivity of compounds for either the intramolecular (telomestatin) or the intermolecular (TMPyP4) G-quadruplex structures. TMPyP4 also down-regulates c-myc and, consequently, hTERT, which may contribute to the inhibition of telomerase-positive cells.

In conclusion, the biological effects of G-quadruplex-interactive compounds, which interact quite preferentially with intramolecular or

[4] D. Sun and W-J. Liu, unpublished observations.

intermolecular G-quadruplex structures, have been investigated. Telomestatin induces and stabilizes intramolecular G-quadruplex structures and prevents them from being disassembled, whereas TMPyP4 preferentially facilitates the formation of and then interacts with intermolecular G-quadruplex structures. Stabilization of intramolecular G-quadruplex structures results in severe damage to the telomere maintenance mechanisms through the inhibition of telomerase activity and induction of S1 nuclease activity, whereas stabilization of intermolecular G-quadruplex structures induces the formation of anaphase bridges. The results from this study provide strong evidence to support our previous suggestion (45, 49) that highly specific and potent G-quadruplex-interactive agents could be promising agents for cancer chemotherapy.

ACKNOWLEDGMENTS

We are grateful to Dr. Kazuo Shin-ya (University of Tokyo, Tokyo, Japan) for providing telomestatin and Dr. Jerry W. Shay (University of Texas, Southwestern Medical Center) for providing the cell lines. We thank Dr. Evonne Rezler for critical reading of drafts of the manuscript and Dr. David Bishop for preparing, proofreading, and editing the final version of the manuscript and figures.

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Mu-Yong Kim, Mary Gleason-Guzman, Elzbieta Izbicka, et al.

Cancer Res 2003;63:3247-3256.