Overexpression and Telomere Capping Alteration* 

G-quadruplex Ligand 12459 Is Associated with Telomerase Resistance to the Short Term Antiproliferative Activity of the 

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Ligands that stabilize the telomeric G-rich single-stranded DNA overhang can be considered as potential anticancer agents that block telomere replication. Ligand 12459, a potent G-quadruplex ligand that belongs to the triazine series, has been previously shown to induce both telomere shortening and apoptosis in the human A549 cell line as a function of its concentration and time exposure. We show here that A549 clones obtained after mutagenesis and selected for resistance to the short term effect of ligand 12459 frequently displayed hTERT transcript overexpression (2–6-fold). Overexpression of hTERT was also characterized in two resistant clones (JFD10 and JFD18) as an increase in telomerase activity, leading to an increase in telomere length. An increased frequency of anaphase bridges was also detected in JFD10 and JFD18, suggesting an alteration of telomere capping functions. Transfection of either hTERT or DN-hTERT cDNAs into A549 cells did not confer resistance or hypersensitivity to the short term effect of ligand 12459, indicating that telomerase expression is not the main determinant of the antiproliferative effect of ligand 12459. In contrast, transfection of DN-hTERT cDNA into resistant JFD18 cells restored sensitivity to apoptotic concentrations of ligand 12459, suggesting that telomerase does participate in the resistance to this G-quadruplex ligand. This work provides evidence that telomerase activity is not the main target for the 12459 G-quadruplex ligand but that hTERT functions contribute to the resistance phenotype to this class of agents.

Telomeres are essential to maintain the stability of chromosome ends and are synthesized by a specialized enzyme called telomerase. Telomerase is overexpressed in a large number of tumors and is involved in cell immortalization and tumorigenesis, whereas it is not described as being expressed in most somatic cells (1). A recent work showed that telomerase was efficiently expressed in S phase from normal cycling cells and played an important function to delay the onset of replicative senescence by maintaining the 3’ telomeric overhang integrity independently from overall telomere length regulation (2). Differential expression of telomerase between normal and cancer cells was the initial rationale for the evaluation of telomerase inhibitors as potential anticancer agents (3), and a highly specific catalytic telomerase inhibitor, BIBR1532, was described as impairing cancer cell proliferation without acute toxicity in a mouse xenograft model (4). Since telomerase is expressed in normal proliferating human cells, the useful therapeutic index of these inhibitors has to be carefully determined in future studies.

Folding of the telomeric G-rich single-stranded overhang into a quadruplex DNA has been found to inhibit telomerase activity. Stabilization of G-quadruplexes can then be considered an original strategy to achieve antitumor activity (5–8). The intracellular existence of G-quadruplexes was recently demonstrated in the telomeres from ciliates (9). G-quadruplex could also be formed from duplex telomeric DNA under appropriate ionic and pH conditions (10) or in the presence of specific ligands (11). The c-myc gene promotor sequence allowed the formation of a G-quadruplex that corresponded to the first demonstration of the physiologic relevance of such a structure in mammalian cells (12).

The 2,4,6-triamino-1,3,5-triazine derivatives are potent telomerase inhibitors that bind to telomeric G-quadruplexes (13). In this series, 12459 (Fig. 1a) is one of the most selective G-quadruplex-interacting compounds and it displayed a 25-fold selectivity when telomerase inhibition was compared with the Taq polymerase inhibition by using the TRAP1-G4 assay (14).

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We dedicate this work to the memory of Professor Claude Hélène (1938–2003).

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1 The abbreviations used are: TRAP, telomere repeat amplification protocol; FISH, fluorescence in situ hybridization; TRF, telomeric restriction fragment; RT, reverse transcriptase; DAPI, 4’,6-diamidino-2-phenylindole; CHAPS, 3-[3-cholamidopropyl]dimethylammonium)-1-propanesulfonic acid; hTERT, human TERT; hTR, human TR; hRNPA1, heterogeneous nuclear ribonucleoprotein A1; Gy, grays.
For apoptosis determination, A549 cells were seeded on 4-well cover slides treated with 12459. Cells were washed with phosphate-buffered saline and stained with Hoechst 33342 at 1 µg/ml. Cells with apoptotic nuclei were counted in a different part of the slide by fluorescence microscopy. Results corresponded to the mean of three separate determinations ± S.D. relative to untreated controls.

Radiation survival was determined by clonogenic assays. Cells were irradiated in culture medium at room temperature in an IBL-687 (137Cs) irradiator (CIS-Biointernational, Saclay, France) at a dose rate of 1.05 Gy/min. Experiments were performed in triplicate or more. Colonies were allowed to grow for 8 days and then fixed with methanol, stained, and scored. Small colonies (<50 cells) were disregarded. Radiation survival curves were drawn for best fit to a linear quadratic model as usual. In S/N2, the hypoxic clonogenic efficiency, S is the residual survival, D is the radiation dose, and α and β are numerical parameters characterizing the radiosensitivity of the cell line.

Infections of A549 and JFD18 Cells—Lentiviral supernatants containing hTERT, DN-hTERT cDNAs, or control HPV vector were a generous gift from Dr. Annelise Bennacerr-Griselli (Institut Gustave Roussy, Villejuif, France). A549 or JFD18 cells at 1.5 × 10⁵ cells/ml were infected at a multiplicity of infection equal to 50 in the presence of 4 µg/ml Polybrene in complete culture medium. Enhanced green fluorescent protein-positive cells were sorted 5 days later by flow cytometry according to a high or low intensity of fluorescence. Populations that expressed a high intensity of fluorescence were sorted in 25-nm² fields and cultivated for up to 81 days. Cytotoxicity and clonogenic survival experiments were performed on cultures between days 60 and 80 after infection.

Clonogenic survival assays were performed with 5 × 10⁴ cells from A549- or JFD18-transfected cell lines according to the previously published procedure [22] in the presence or absence of 5 µM 12459. Results represent the mean value ± S.D. of triplicate determinations.

RNA Preparation and RT²-PCR Assays—Total RNA was isolated from 1 × 10⁶ cells using Tri-Reagent (Sigma) as recommended by the manufacturer. One µg of total RNA was reverse transcribed in a 20-µl reaction volume using random hexamers, avian myeloblastosis virus reverse transcriptase, and the reaction buffer provided in the reverse transcription kit (Promega). The volume of the sample was adjusted to 200 µl with diethylpyrocarbonate-treated water at the end of the reaction. A 10-µl aliquot of cDNA was used for PCR amplifications. hTERT was amplified using the forward TERT2109 primer (5'-GGCTGAAGCT-GAACATTGTCA-3') and the reverse TERT2631 primer (5'-AGGCT- GCCAGACGCGGAGAAGG-3') according to Ref. 23, 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. Amplification of β2-microglobulin was performed as a control using the same PCR conditions with primer forward (5'-ACCCCACGTTAAAAGAATGTA-3') and primer reverse (5'-ATCCCTAACAATCTCAGCATG-3'). Amplifications of the different 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. Amplification of β2-microglobulin was performed as a control using the same PCR conditions with primer forward (5'-ACCCCACGTTAAAAGAATGTA-3') and primer reverse (5'-ATCCCTAACAATCTCAGCATG-3'). Amplifications of the different genes were performed using the same PCR conditions with primer forward (5'-ACCCCACGTTAAAAGAATGTA-3') and primer reverse (5'-ATCCCTAACAATCTCAGCATG-3') for each of the following primers: hTR, forward primer (5'-CTAAACCTAATCTGAGAAGGCGGTAG-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); TRF1, forward primer (5'-AGCAAGAAGGGCCTACCGAAAGCCA-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); TRF2, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); WRN, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); NCL, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); BLM, forward primer (5'-GCTTCCAGAAATCTCCAGCACC-3') and reverse primer (5'-GCTTCCAGAAATCTCCAGCACC-3'); WRN, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); NCL, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); BLM, forward primer (5'-GCTTCCAGAAATCTCCAGCACC-3') and reverse primer (5'-GCTTCCAGAAATCTCCAGCACC-3'); WRN, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); NCL, forward primer (5'-CTTCCAAGAAATCTCCAGCACC-3') and reverse primer (5'-GTTTGGTCTGATTAAGTCACCCAACTG-3'); BLM, forward primer (5'-GCTTCCAGAAATCTCCAGCACC-3') and reverse primer (5'-GCTTCCAGAAATCTCCAGCACC-3'). Amplified products were resolved on 6% nondenaturing polyacrylamide gels in 1X TBE and stained with SYBR Green I (Roche Applied Science). Quantitation was performed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and software analysis, relative to the signal of β2-microglobulin. Results represent the mean value of two or three independent RNA extractions, as indicated.

Telomeric Restriction Size Fragment Determination—Genomic DNA was digested with HinfI/RsaI restriction enzymes and electrophoresed in 0.8% agarose gels in 1X TBE. DNA was transferred onto nylon membrane (Amersham Biosciences) and then prehybridized for 2 h at 65 °C in 7% SDS, 1% bovine serum albumin, and 0.5 µM NaPO₄, pH 8.0. A 0.7-kb telomere DNA probe (pUCTeo; a gift from Prof. E. Gilson, Ecole Normale Superieure, Lyon, France) was labeled with α-³²P-dCTP and hybridized to the telomeric probe. Autoradiograms were scanned, and the sizes of the telomeric probes were determined. Telomere sizes were normalized to the size of the 6.7-kb probe, which represents the average telomere size in human lymphocytes.
hybridization was performed overnight at 65 °C. Membranes were washed twice with 0.2× SSC, 0.1% SDS at 65 °C for 15 min and then twice with 2× SSC, 0.1% SDS at room temperature. Telomeric smears were revealed by autoradiography, and the mean length of the telomere restriction fragment (TRF) corresponds to the peak of the densitometric integration curve from two separate experiments.

FISH Analysis—Metaphase chromosome spreads were prepared from cells treated with colcemid (0.1 μg/ml, 1 h; Eurobio) and then hypotonic KCl and fixed in methanol/acetic acid (3:1, v/v). Fixed cells were dropped onto clean slides and left to dry overnight prior to hybridization with a telomeric specific (CCCTAA)3-Cy3 PNA probe (PerSeptive Biosystems) as described (24) and staining with DAPI. Fluorescent signals were visualized under a UV microscope (Axioplan²; Zeiss) equipped with a computer-piloted filter wheel and were captured with a CCD camera (Photometrics-Sensys) using the Smart-Capture software (Vysis) (settings: gain 3 (red), 1 (blue); binning 4) and a fixed exposure time of 2 s. A flat field template was used to correct for unevenness in field illumination. Merged DAPI-Cy3 pseudocolor images were used to colocalize chromosomes and telomere signals. Original grayscale Cy3 images were saved for quantitative analysis using the Iplab Spectrum P software (Skanalytics). Overall telomere fluorescence was estimated by calculating the mean pixel value of the metaphase using an automatic segmentation protocol provided by the software.

Anaphase Bridge Analysis—To determine the presence of anaphase bridges, cells were seeded on microscope slides and stained with Chromomycin A3 (Sigma). Images of anaphases were recorded with a confocal microscope (Bio-Rad MRC 1024) with excitation at 457 nm and emission at 530 nm. At least 50 metaphases were examined for sensitive A549 and JFD10- or JFD18-resistant clones.

**RESULTS**

Resistant clones were obtained after ethyl methyl sulfonate mutagenesis and soft agar cloning selection (26) in the presence of 10 μM 12459 (Fig. 1b), a concentration of drug able to induce apoptosis with a 72–96-h delay (Fig. 2a). After the initial selection procedure, clones were plated and maintained in the absence of 12459. Among 200 clones isolated, 15 were confirmed for 12459 resistance in a 96-h cytotoxicity assay with resistance indexes varying from 3- to 5-fold (Fig. 2b). The resistance phenotype of the selected clones is stable for at least 6 months, when cells are grown in the absence of 12459.

The level of hTERT transcripts was investigated in the resistant clones by RT-PCR analysis. hTERT presents a complex isothiocyanate-conjugated antidigoxigenin antibody (Sigma), respectively. Chromosomes were counterstained with DAPI, and fluorescent signals were visualized and captured as above. Merged DAPI-fluorescein isothiocyanate-Texas Red images were then obtained to count and localize hTERT loci.

**FIG. 1.** Selection of A549 clones resistant to the G-quadruplex ligand 12459. a, chemical structure of 12459. b, schematic diagram of the resistance selection process.

**FIG. 2.** Antiproliferative and apoptotic effects of 12459. a, apoptosis induction by 12459 in A549 cells. Cells were treated for 24, 48, 72, and 96 h with 12459 at 10 μM. Cells were fixed and stained with Hoechst 33342, and the percentage of cells exhibiting apoptotic nuclei was calculated relative to untreated cells. b, antiproliferative effect of 12459 against parental A549 and resistant JFD10 and JFD18 clones for 96-h drug exposure.
splicing pattern that includes an active (+α, +β) transcript and several inactive species including one major (+α, +β) transcript and two minor (−α, −β) and (+α, −β) hTERT transcripts. The size of the PCR products (bp) is indicated on the right. c. Quantification of three independent RT-PCR experiments (from independent RNA extractions) for the active (+α, +β) and the inactive (−β) hTERT transcripts on A549 parental cells and JFD10-, JFD18-, JFD9-, and JFD11-resistant clones. Data were normalized relative to the β2-microglobulin transcript (β2m) and to the values of parental A549 cells defined as 1.

In order to determine whether the increased hTERT transcript levels resulted in an increase in telomerase activity, TRAP activity was measured on serial amounts of protein extracts prepared from A549 and JFD18 cells. In agreement with its (+α, +β) hTERT mRNA increase, telomerase activity was found augmented in JFD18 and JFD10 cells as compared with parental cells (Fig. 6a). In contrast, JFD9 and JFD11 did not present variations of telomerase activity, as compared with A549 cells (results not shown). The in vitro inhibitory effect of 12459 was also measured by the TRAP-G4 assay (14) on extracts from A549 and JFD10-and JFD18-resistant cells. 12459 and telomestatin were found to inhibit TRAP-G4 with equal IC50 values for sensitive and resistant extracts (results not shown). These results excluded qualitative alterations of telomerase that could modify the sensitivity of the enzyme to the in vitro effect of these inhibitors.

Treatment of A549 cells with 12459 was previously shown to down-regulate telomerase activity (13). We have determined whether resistance phenotype is altering the effect of 12459 to down-regulate telomerase activity in JFD10 and JFD18 clones. As shown in Fig. 6b, telomerase activity measured by TRAP from A549-treated cells was strongly decreased. In contrast, telomerase activity measured by TRAP in JFD10 and JFD18 cells under 12459 treatment remained detectable with levels comparable with that from untreated resistant cells (Fig. 6b and c), thus indicating that JFD10 and JFD18 are resistant to the 12459-induced down-regulation of telomerase activity.

**Fig. 3.** Expression of hTERT transcript in 12459-resistant clones. a, RT-PCR of hTERT in A549 cells and JFD-resistant clones, as indicated. +α, +β, RT-PCR of hTERT in A549 and JFD10-, JFD18-, JFD9-, and JFD11-resistant clones, as indicated. The β2-microglobulin transcript is used as a control for mRNA expression. RT-PCR analysis detects active (+α, +β) and inactive (−β) hTERT transcripts. The size of the PCR products (bp) is indicated on the right. c. Quantification of three independent RT-PCR experiments (from independent RNA extractions) for the active (+α, +β) and the inactive (−β) hTERT transcripts on A549 parental cells and JFD10-, JFD18-, JFD9-, and JFD11-resistant clones. Data were normalized relative to the β2-microglobulin transcript (β2m) and to the values of parental A549 cells defined as 1.
An expected consequence for the clones that presented increased \((+/\alpha, +/\beta)\) hTERT transcript levels is a gain in telomere length. We analyzed the telomere length in JFD10 and JFD18 after 3 months in culture in the absence of 12459. These two clones presented a mean length TRF of 8–9 kb, as compared with 6.5 kb measured in the parental A549 cells (Fig. 7a). Quantitative FISH analysis also indicated a 42% increase in the PNA probe hybridization intensity at telomeres for the JFD18 clone, as compared with A549 (Fig. 7b and c). Altogether, these data are consistent with an increase in telomerase activity in these clones due to hTERT overexpression. FISH analysis showed three copies of the chromosome 5, each bearing one copy of the hTERT gene in both A549 and JFD18 metaphases, suggesting that the hTERT overexpression did not result from locus amplification or chromosome duplication (Fig. 7d).

The presence of increased anaphase bridges has been associated with a disruption of the capping function of the telomere (28, 29). Also, another G-quadruplex ligand, TMPyP4, has been found to induce anaphase bridges in sea urchin oocytes (30). We have determined whether 12459 may induce the formation of anaphase bridges in A549 and resistant clones. We were not able to detect a significant presence of anaphase bridges \((\leq 5\%)\) for concentrations of 12459 ranging from 0.1 to 10 \(\mu\)M after a 48-h treatment with the drug in both sensitive and resistant cells (not shown). In contrast, untreated JFD10- and JFD18-resistant cells presented a higher basal level of anaphase bridges \((25–30\%)\) as compared with the A549-sensitive cell line \((<5\%)\). Typical images of anaphase bridges obtained with A549, JFD10, and JFD18 cell lines are shown in Fig. 8a. In the JFD18 clone, FISH analyses also revealed occasional telophase bridges, in the middle of which telomere signals are detected (Fig. 8b). Interestingly, this high incidence of anaphase bridges has no apparent effect on the proliferation rate of the resistant cells, since their doubling time remained equivalent to that of A549 cells \((20–21\) h). We concluded that selection of resistance has altered telomere capping functions without perturbing cell division.

The JFD18 clone presented a 5-fold resistance to 12459 (Fig. 2a) and was further evaluated for its cross-resistance pattern to other G-quadruplex ligands or to other cytotoxic agents with
various mechanisms of action. As indicated in Fig. 9a, JFD18 has no cross-resistance to the topoisomerase inhibitors doxorubicin, etoposide, and camptothecin and to the G-quadruplex ligands telomestatin and BRACO19. In contrast, JFD18 displayed a partial cross-resistance to the DNA-interactive agent mitomycin C (3.7-fold) and to the tubulin poison vinblastin (2.1-fold) and was slightly cross-resistant to the triazine derivative 115405 (1.8-fold). These data indicated that JFD18 does not have the characteristics of a multidrug resistance phenotype. In agreement, no variation in multidrug-related protein 1 (MRP1) and breast cancer resistance protein (BCRP) transcripts was found in JFD18, and the multidrug resistance 1 (MDR1) transcript remained undetectable (Fig. 5b). Similar results were found for the JFD10 clone that displayed a 5-fold resistance to 12459 (Fig. 5b). It should be noticed that non-triazine G-quadruplex ligands displayed poor growth-inhibitory properties against A549 cells, as compared with triazine derivatives, with IC₅₀ values equal to 9 and 3 μM for telomestatin and BRACO19, respectively. Furthermore, JFD18 cells did not present significant resistance against the effect of ionizing radiation, as compared with A549 (Fig. 9b).

Analysis of hTERT expression in the different 12459-resistant clones suggested that two phenotypic classes of clones were observed. In the first, active hTERT transcript was up-regulated (i.e. JFD10 and JFD18), and in the second, active hTERT transcript was maintained at the same levels as compared with A549 (i.e. JFD9 and JFD11). These results raised the question of whether or not an increased telomerase activity is a key element in the resistance to short term treatment with 12459 in A549 cells. To address this possibility, we investigated the effect of 12459 in A549 cells transfected either with hTERT or with DN-hTERT cDNA and in JFD18 cells transfected with DN-hTERT cDNA. As expected, expression of hTERT in A549 cells considerably increased telomerase activity and telomere length, as compared with cells transfected with an empty vector (not shown). On the other hand, the expression of DN-hTERT abolished the telomerase activity in both A549 and JFD18 cells (not shown), as already described for this mutant (31, 32). After 80 days of culture, the DN-hTERT A549 and DN-hTERT JFD18 cells both showed a significant decrease in telomere length (Fig 10). This suggested that the overexpression of telomerase in JFD18 cells was necessary to maintain the telomere length and that telomere lengthening in JFD18 was not due to a telomerase-independent mechanism selected during resistance acquisition.

The short term antiproliferative activity of 12459 was determined in cells transfected with the different constructions in order to evaluate the effects of overexpression or inactivation of telomerase. Interestingly, hTERT A549 or DN-hTERT A549 cells did not present significant differences in their sensitivity.
to the antiproliferative activity of 12459, as compared with HPV A549 cells transfected with the empty vector (not shown). These results suggested that variations of telomerase activity were not essential for the sensitivity of A549 cells to 12459. On the other hand, DN-hTERT JFD18 cells displayed an increased sensitivity for the highest concentrations of 12459 assayed (10 and 30 μM), as compared with HPV JFD18 cells (Fig. 11a). This suggests that interfering with telomerase activity in these cells partially restores the sensitivity to 12459 for concentrations of compound equivalent to that used during the resistance selection procedure.

To confirm these results, we determined the effect of 12459 on the transfected cell line in a soft agar clonogenic survival assay. In the presence of 5 μM 12459, we found identical clonogenic survivals for HPV A549, hTERT A549, and DN-hTERT A549 (Fig. 11b). On the other hand, the full resistance to the effect of 12459 for clonogenic survival of the HPV JFD18 cell line was found to be almost completely reversed in the DN-hTERT JFD18 cell line (Fig. 11b).

**DISCUSSION**

We have described in the present work the characterization of A549 clones selected for resistance to 10 μM 12459. A characteristic of the majority of the clones is an overexpression of the hTERT transcript that varies from 2- to 6-fold. The increased hTERT transcript level is related to an increase in telomerase activity in two of the hTERT overexpressing clones, JFD10 and JFD18, and as a consequence these clones also present longer telomeres (28). The establishment of resistance to 12459 in A549 cells by using a different procedure (i.e., lower 12459 concentration and long term senescence as a selection criterion to obtain the
JFA2-resistant cells) also led to a 2-fold increase in hTERT transcript levels, an increase in telomere lengths, and higher incidence of anaphase bridges (33). Therefore, up-regulation of telomerase expression and telomere capping modification represent frequent phenotypic alterations related to resistance to this G-quadruplex ligand.

On the other hand, telomerase overexpression in these resistant models may not necessarily indicate that there is a direct link between levels of telomerase activity and the cellular effects of these ligands. Any pathway that may antagonize the drug effect in resistant cells is expected to restore normal telomerase activity. However, our results indicated that drug inactivation is not mediated by multidrug resistance genes and that JFD18 does not have the cross-resistance characteristics of a multidrug-resistant cell line.

A more direct argument indicating that modulation of telomerase activity is not a major determinant for the antiproliferative activity of 12459 was obtained by the transfection experiments with hTERT or DN-hTERT in A549 cells in which antiproliferative activity was not modified. This agrees with previous results showing that triazine derivatives were able to inhibit proliferation of telomerase-positive and -negative cell lines, including the GM847 ALT cell line (13). Similar findings were recently published for other G-quadruplex ligands, such as the porphyrin derivative TMPyP4 that was found to be active against telomerase-positive and -negative cell lines (11). In contrast, telomestatin was found to be active against telomerase-positive cells but inactive against ALT cells (11). This study also suggested a link between the selectivity of telomestatin for intramolecular G-quadruplex or TMPyP4 for intermolecular G-quadruplex and the ability of these compounds to mediate different biological effects against telomerase-positive or -negative (ALT) tumor cell lines (11). In contrast to these compounds, 12459 was not found to distinguish between intramolecular, dimer, and intermolecular forms of quadruplexes, a result that may explain the biological differences between 12459, telomestatin, and TMPyP4.

Although transfection of hTERT was not sufficient to confer resistance to 12459 in A549 cells, our results demonstrated that increased telomerase activity participated in the mechanism of resistance to 12459. The expression of DN-hTERT in the JFD18-resistant clone reversed the resistance to concentrations of 12459 able to induce apoptosis in A549 cells.

It has been suggested that the induction of quadruplex formation in telomeric overhang repeats would inhibit telomerase activity and therefore telomere stabilization (34). End-capping of telomeres is controlled by telomerase itself (35, 36) and by TRF2, a double-stranded telomeric DNA-binding protein that participates in the formation of T-loop structures at telomeres.
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(37) Overexpression of TRF2 protects critically shortened telomeres from fusion and delays the onset of replicative senescence (38). The human protein hPot1 that specifically binds to the single-stranded 3’ overhang of telomere sequence plays a potential role in telomere protection. Deletion of the hPot1 homologue in yeast results both in the loss of telomeric DNA and in end-to-end fusions (39). Pot1 was recently shown to modulate telomere elongation by telomerase (40, 41) and may serve as a terminal transducer of TRF1, a negative regulator of telomerase activity (40). The human homologue of EST1 that recruits or activates telomerase at the 3’ end of telomeres, hEST1A, also affects telomere capping when overexpressed (28).

It is suspected that ligands that stabilize the folding of single-stranded telomeric DNA overhang into stable quadruplex structures might effectively compete with the end-capping functions of hPot1, TRF2, or telomerase itself, with dramatic rapid and rapid consequences for cell viability (34). Our results indicated that telomerase is necessary for the maintenance of resistance in JFD18 cells, but it is not sufficient to induce resistance in sensitive cells. An attractive explanation for such a difference would be a modification of the telomere capping in resistant cells in which the need for telomerase capping functions became essential to maintain efficient telomere protection against the effect of 12459. This change is reflected by the increased rate of anaphase bridge formation in the resistant clones. It is also possible that telomerase activity is indirectly modulated by the alteration of another factor essential for telomere end-capping.

Recent reports have also shown that telomerase overexpression can suppress DNA damage and/or damage-related signals that trigger cell death (42–44). This may represent an alternative explanation for the resistance to apoptotic concentrations of 12459. However, the cross-resistance profile of the JFD18 clone discriminated between a DNA-damaging agent such as mitomycin C, found to be cross-resistant, and between DNA topoiso merase inhibitors or ionizing radiations that were not. In the JFD9 clone that does not overexpress telomerase, mitomycin C was found to be 2.7-fold cross-resistant, suggesting rather a telomerase-independent cross-resistance pathway for JFD18 and JFD9 for this DNA-damaging agent. A detailed analysis of the apoptotic pathways for these clones would give interesting clues on these points.

In conclusion, our work presents evidence that the level of telomerase activity is not directly linked to the antiproliferative activity of 12459 but that resistance to this G-quadruplex ligand is frequently associated with both up-regulation of telomerase activity and alteration of telomere capping functions that may participate directly or indirectly in the mechanism of resistance in some clones.

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REFERENCES
1. McEachern, M. J., Krauskopf, A., and Blackburn, E. H. (2000) Annu. Rev. Genet. 34, 331–358
2. Masutomi, K., Yu, Y. E., Khurts, S., Ben-Porath, I., Currier, J., Metz, G. B., Brooks, M. W., Kaneto, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., and Hahn, W. C. (2003) Cell 114, 241–253
3. Sharma, S., Raymond, E., Soda, H., Sun, D., Hilsenbeck, S. G., Sharma, A., Ibicka, E., Windle, B., and Von Hoff, D. D. (1997) Oncol. 8, 1063–1074
4. Damm, K., Hemmann, U., Garnin-Chessa, P., Haeu, N., Kauffman, I., Priepke, H., Niess, J., Catter, D., Chen, K., Li, C., and Muller, M. (1999) Oncogene 18, 2495–2500
5. Parkinson, G. N., Lee, M. P., and Neidle, S. (2002) Nucleic Acids Res. 30, 4364–4371
6. Mergny, J. L., Riou, J. F., Mailliet, P., Teulade-Fichou, M. P., and Mattson, M. P. (1999) J. Biol. Chem. 274, 7264–7271
7. Knipe, D. M., and Hurley, L. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 515–520
8. Schaffitzel, C., Berger, I., Postberg, J., Hanes, J., Lipps, H. J., and Pluckthun, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7876–7881
9. Akiyama, M., Yamada, O., Kanda, N., Akita, S., Kawano, T., Ohno, T., Mizoguchi, S., Hayakawa, Y., and Seto, H. (2001) J. Am. Chem. Soc. 123, 1262–1263
10. Kim, M. Y., Gleason-Guzman, M., Ibicka, E., Nishida, D., and Hurley, L. H. (2000) Cancer Res. 60, 3247–3256
11. Siddiqui-Jan, A., Grand, C. L., Barnes, D. J., and Hurley, L. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1573–1578
12. Gomez, D., Mergny, J. L., and Riou, J. F. (2002) Cancer Res. 62, 3365–3368
13. Zhang, X., Mar, V., Zhou, W., Harrington, L., and Robinson, M. O. (1999) Genes Dev. 13, 2388–2399
14. Orlando, I., and Liu, J. J. (2002) Nucleic Acids Res. 30, 4618–4625
15. Kim, M. Y., Gleason-Guzman, M., Ibicka, E., Nishida, D., and Hurley, L. H. (2000) Cancer Res. 60, 3247–3256