# Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands

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**Telomeres of human chromosomes contain a G-rich 3′-overhang that adopts an intramolecular G-quadruplex structure *in vitro* which blocks the catalytic reaction of telomerase. Agents that stabilize G-quadruplexes have the potential to interfere with telomere replication by blocking the elongation step catalyzed by telomerase and can therefore act as antitumor agents. We have identified by Fluorescence Resonance Energy Transfer a new series of quinoline-based G-quadruplex ligands that also exhibit potent and specific anti-telomerase activity with $IC_{50}$ in the nanomolar concentration range. Long term treatment of tumor cells at subapoptotic dosage induces a delayed growth arrest that depends on the initial telomere length. This growth arrest is associated with telomere erosion and the appearance of the senescent cell phenotype (large size and expression of β-galactosidase activity). Our data show that a G-quadruplex interacting agent is able to impair telomerase function in a tumor cell thus providing a basis for the development of new anticancer agents.**

telomerase inhibitor | tetraplex | drug–DNA recognition

The reactivation of telomerase activity in most cancer cells supports the concept that telomerase is a relevant target in oncology, and telomerase inhibitors have been proposed as new potential anticancer agents (1, 2). Most of human telomeric DNA is double-stranded and contains $(TTAGGG/CCCTAA)_n$ repeats, except for the extreme terminal part, which involves a G-rich 3′ overhang (3). This sequence may adopt an intramolecular G-quadruplex structure *in vitro* (also called tetraplex) that blocks the catalytic reaction of telomerase (ref. 4; see Fig. 1 *A* and *B*). Recent reports emphasize that specific recognition of G-quadruplexes may be achieved (5–12). Agents that stabilize G-quadruplexes have the potential to interfere with telomere replication by blocking the elongation step catalyzed by telomerase (6) and can therefore act as antitumor agents (13–17). We have designed a fluorescence resonance energy transfer (FRET) assay to identify such G-quadruplex ligands (G4 FRET; refs. 18, 19). The melting temperature of a quadruplex-forming oligonucleotide was measured in the presence of different molecules. Different chemical series of G4 ligands have subsequently been identified. In this report, some selected analogues of a novel 2,4,6-triamino-1,3,5-triazine series exhibited interesting properties (Fig. 1*C*). The ligand-induced stabilization of the quadruplex was associated with potent inhibition of telomerase activity, telomere shortening, and delayed induction of senescence in human telomerase-positive cells.

## Experimental Procedures

**Oligonucleotides and Compounds.** All oligonucleotides were synthesized and purified by Eurogentec, Seraing, Belgium. The synthesis of the triazine derivatives will be presented elsewhere. Solutions of all derivatives were prepared at 10 mM in DMSO and were kept at −20°C in the dark between experiments. Further dilutions were made in water.

**UV or Fluorescence Melting Experiments and Fluorescence Titrations.** All measurements were performed as described (18–20).

**Assay of Telomerase Activity and *Taq* Polymerase Assay.** Telomerase activity was assayed using a modified telomere repeat amplification protocol (TRAP) assay (21, 22). The specificity of compounds was assayed with the *Taq* polymerase reaction by using the polylinker from plasmid pCDNA1 as a DNA template (23). The telomerase inhibitory effect of triazines on cultured A549 cells originating from a human lung carcinoma was measured after 24 h drug treatment, on total cell extract (24). Briefly, cells ($10^6$ cells per culture) were treated for 24 h in complete culture medium, then washed three times in 1× PBS. Cells were scraped in PBS, pelleted by centrifugation for 5 min at 400 × *g*, and resuspended in 200 μl of lysis buffer that contained 0.5% CHAPS, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, 10% glycerol, and 10 mM Tris·HCl (pH 7.5). The lysate was incubated for 30 min at 4°C and centrifuged at 12,000 × *g* for 20 min at 4°C, and protein concentration determined using a Bio-Rad kit assay. Telomerase activity was determined on aliquots of 20 and 200 ng of protein extract by TRAP assay, for each concentration of triazine, each point in triplicate. Quantification of telomerase activity was determined by using an Instantimager (Packard). Values are expressed as percent of telomerase inhibition relative to control untreated cells. In some indicated experiments an internal control (ITAS) corresponding to the 36-mer (5′-AATCCGTCGAGCAGAGT-TAAAAGGCCGAGAAGCGAT-3′) was added according to ref. 25.

**Cell Culture.** All cell lines, except hTERT-BJ1 (26), GM847DM (27), and MRC5-V1 (28) were from American Type Culture Collection. Antiproliferative activity by triazines was performed as described (29). For apoptosis determination, A549 cells were plated on 4-well Sonicseal slides (Nunc) and treated with triazines. Cells were washed with PBS and stained with Hoechst 33342 at 1 μg/ml. Cells with apoptotic nuclei were counted in the different part of the slides by using an Olympus UV BX60 fluorescence microscope (New Hyde Park, NY). Results, corresponding to the mean of triplicate determination (SD <10%), are expressed relative to control untreated cells.

For long term growth of A549 cells, triazine-treated or untreated cells were seeded at $0.9 \times 10^6$ cells into 125 $cm^2$ tissue

Abbreviations: TRAP, telomere repeat amplification protocol; FRET, fluorescence resonance energy transfer; TRF, telomeric restriction fragment.

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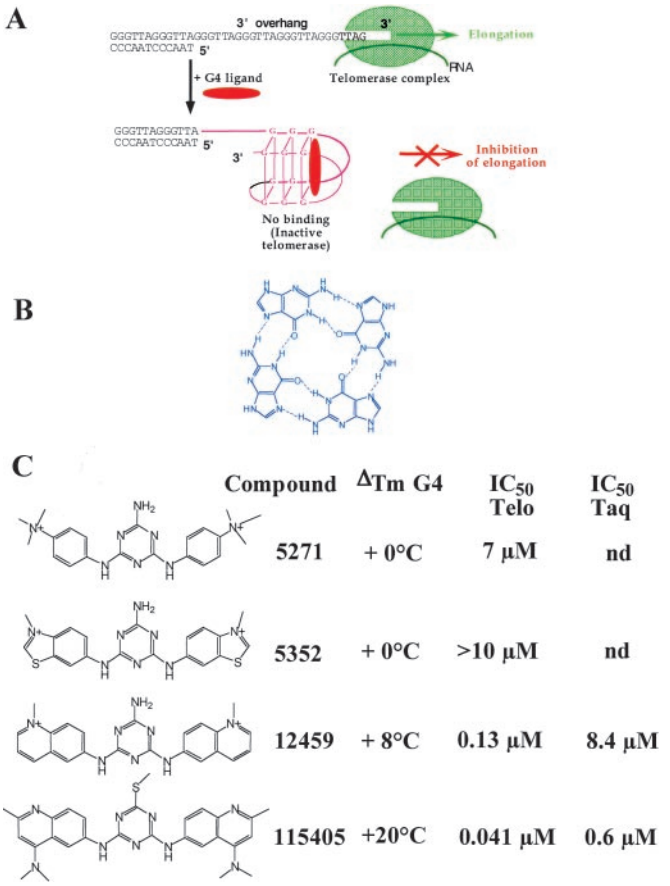


**Fig. 1.** Presentation of the system. (*A*) Possible mechanism of telomerase inhibition by G-quadruplex induction. (*B*) Structure of the G-quartet (G4) also known as a G- or $G_4$-tetrad. (*C*) Chemical formula of triazine derivatives tested as G4 ligands. ΔTm (°C) values measured by G4 FRET assay. $IC_{50}$ values measured in telomerase assay (Telo) and in *Taq* polymerase assay (Taq).

culture flask for 3 or 4 days, then trypsinized and counted. Each time, $0.9 \times 10^6$ cells were replated onto new culture flask with fresh triazine solution. The rest of the cells in each passage were pelleted to prepare genomic DNA or replated to prepare chromosome spread or β-galactosidase assay. For long-term growth of hTERT-BJ1 cells, triazine-treated or untreated cells were seeded at $0.5 \times 10^6$ cells into 75-$cm^2$ tissue culture flasks for 3 or 4 days, then trypsinized and counted. Treatments were done in duplicates.

**β-Galactosidase Activity.** A549 cells were plated in 4-well Sonicseal slides (Nunc) and grown for 48 h. Medium was removed and cells were washed in PBS, and fixed in 1% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. After two washes in PBS, cells were incubated for 12 h with β-galactosidase stain solution containing 0.4 mg/ml X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS.

**Telomeric Restriction Fragment (TRF) and Fluorescence *in Situ* Hybridization (FISH) Assays.** Genomic DNA was digested with *Hae*III/*Hin*f I and electrophoresed in 0.8% agarose gels in TBE buffer. After electrophoresis, denaturation and hybridization were performed directly on the gel by using a $^{32}$P-labeled $(C_3TA_2)_3$ probe as described (30). Telomeric smears were visualized on Instantimager (Packard) and the mean length of the TRFs that corresponds to the peak of the integration curve was measured relative to DNA molecular weight markers (27).

Treated or untreated A549 cells were harvested at 35 population doublings following colchicin incubation (1.5 μg/ml for 3 h). After hypotonic swelling in 75 mM KCl (15 min at 37°C), cells were fixed and stored in ethanol/acetic acid. Before hybridization, cells were dropped on slides and dried overnight. After washing with PBS, slides were denatured by formamide (70%) in 4× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) at 75°C for 3 min, then dehydrated in ice cold ethanol and air dried. Hybridization mixture (30 μl) containing 50% formamide, 10% dextran sulfate, 4× SSC, 0.25% blocking reagent (DuPont), 0.6 μg/ml Cy3-conjugated probe was added to the slide, covered with a coverslip, and followed by DNA denaturation (10 min at 75°C). After hybridization for 12 h at 37°C, slides were washed with 50% formamide and 2× SSC (5 min at 45°C), then with 0.1× SSC (5 min at 60°C) and with 4× SSC containing 0.05% Tween 20 (at room temperature). Slides were dehydrated with ethanol, air dried, and covered by antifade solution containing 4′,6-diamidino-2-phenylindole (DAPI, Stratagene). Modified Cy3-$(C_3TA_2)_3$ telomeric probe containing 2′-OMe ribose sugars and 5-(1-propynyl)pyrimidine residues (31) was synthesized by Eurogentec. Images were acquired by using a Nikon Microphot microscope. Telomeric spots were analyzed on at least six individual metaphases and results were expressed as percent of chromosomes that contain 0, 1, 2, 3, or 4 detectable telomeres.

## Results and Discussion

A number of small molecules have been discovered to inhibit the function of telomerase by stabilizing G4-DNA structures (10–14). Triazines were compared at 1 μM dye concentration, and the results are summarized on Fig. 1*C*. The best ligand **1150405** gave a ΔTm of +20°C, followed by **12459,** which gave a ΔTm of + 8°C, whereas compounds **5271** and **5352** did not stabilize the G-quadruplex. Similar Tm values were obtained for these molecules when a G4 FRET assay was performed in the presence of a hundred-fold excess of double-stranded DNA (18), indicating a good specificity for G-quadruplexes. This specificity was confirmed by equilibrium dialysis experiment (32) (to be presented elsewhere). The stabilization obtained with **115405** compares favorably with all G4-ligands tested so far (18, 19, 23, 33).

The ΔTm effect was compared with telomerase inhibition *in vitro*. The **115405** and **12459** compounds displayed potent telomerase inhibitory activity when evaluated in a telomerase TRAP assay. Quinoline-substituted triazines, such as **115405** and **12459**, were the most efficient inhibitors with $IC_{50}$ (telo) of 41 nM and 130 nM, respectively, whereas **5271** and **5352** were found to be weakly active and inactive, respectively, in good agreement with the absence of G-quadruplex stabilization (Fig. 1*C*). Judging from telomerase inhibition and FRET assays, the two active compounds are considered to be very efficient inhibitors. In general, a good correlation was found between G-quadruplex stabilization potency and telomerase inhibition among the one hundred triazine derivatives synthesized (data not shown; described in patent WO 0140218). To discriminate between telomerase elongation inhibition and *Taq* polymerase inhibition during the amplification steps of the assay, the compounds were tested independently with *Taq* polymerase and a DNA substrate unable to fold into G-quadruplexes. *Taq* polymerase was inhibited but at higher concentrations (Fig. 1*C*). $IC_{50}$s for *Taq* inhibition were 610 nM and 8400 nM for **115405** and **12459**, respectively. Inclusion of an internal control to the TRAP assay also confirmed these results (ITAS, Fig. 2*a*). No inhibition of ITAS was detected up to 3 μM. Furthermore, only a weak inhibition of telomere products was detected if the compound was added to the mixture between telomerase extension and PCR amplification. Therefore telomerase inhibition,

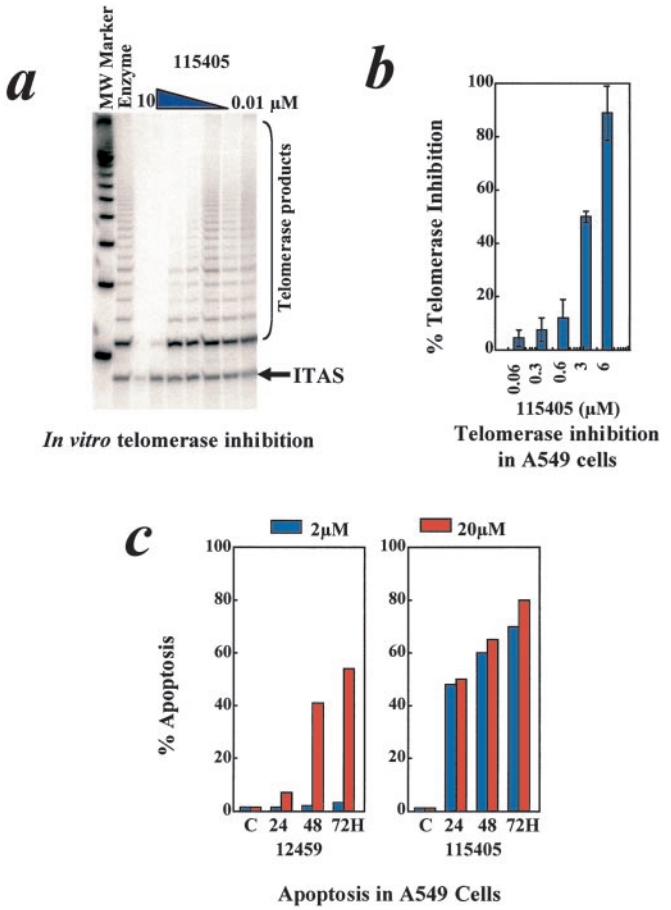


**Fig. 2.** TRAP inhibition and short-term cellular properties of triazines. (*a*) *In vitro* telomerase inhibition by **115405**. Decreasing concentrations of **115405** [10–0.01 $\mu$M were added in a TRAP assay containing an internal standard (ITAS) (25)]. Inhibition of ITAS (*Taq* polymerase activity) is observed at higher concentrations than for telomerase inhibition (Telomerase products). (*b*) Telomerase inhibitory effect of **115405** on cultured A549 cells originating from a human lung carcinoma. TRAP activity was determined on A549 cell extracts (200 ng) after 24-h treatment with different concentrations of **115405**. Values (triplicate determination ± SD) are expressed as percent inhibition of telomerase relative to untreated controls. The addition of active telomerase to treated samples allowed activity recovery, indicating that telomerase inhibition may result from indirect down-regulation. (*c*) Apoptosis induction by triazine derivatives in A549 cells. Cells were treated for 24, 48, and 72 h with either **12459** or **115405** at 2 and 20 $\mu$M. Cells were fixed and stained with Hoechst 33342 and the percentage of cells exhibiting apoptotic nuclei was calculated relative to untreated cells.

rather than *Taq* inhibition, is responsible for the observed effect on TRAP.

The *in vitro* potency of these triazine derivatives prompted us to investigate their effects in cultured cells. Ligand **115405** induced a dose-dependent decrease in telomerase activity relative to untreated cells (Fig. 2*b*) with an $IC_{50}$ (telo) of 3 $\mu$M, for a 24 h treatment. A lower inhibition of telomerase activity was induced by **12459**, whereas no inhibition was detected for **5271**, in agreement with their relative potency *in vitro*. The G-quadruplex-stabilizing compounds were also active as antiproliferative agents on a panel of one human cancer cell lines and three immortalized human cell lines (Table 1). Ligand **115405** was active on the whole panel (IC50 $\leq$ 2 $\mu$M), including the ALT (Alternative Lengthening of Telomere) cell line GM847DM (27), the SV40 immortalized lung fibroblast cell line MRC5-V1 (28), and the telomerase immortalized fibroblast hTERT-BJ1 cell line (26). Ligand **12459** was active against only two cell lines, and **5271** and **5352**, were devoid of significant antiproliferative activity. Antiproliferative properties were correlated with the *in vitro* stabilization of G-quadruplexes, in contrast with other published G4 ligands (10, 34). Ligand **115405** was found to induce major apoptosis within 24 h at micromolar concentrations in A549 cells (Fig. 2*c*). Recent reports indicate that short-term and massive apoptosis may result from interference with telomere function when either hTERT or hTR are modified by mutations (35, 36). The short-term apoptosis observed here may either result from an effect on telomeres or a nonselective effect on other DNA targets at micromolar concentrations. Further studies aimed at determining the causes of apoptosis have been undertaken through a microarray approach and will be presented elsewhere.

We then examined whether G-quadruplex ligands could interfere with telomere replication. Long-term treatment at subcytotoxic doses of telomerase-positive cultured cells was therefore undertaken, using cell lines of various initial telomere length. We hypothesized that if one part of the mechanism of action of these agents is linked to G-quadruplex interaction at telomeres, we would observe a delayed impact on telomere length. To avoid short-term apoptosis and other nonspecific events due to the narrow selectivity of these compounds that could render the detection of telomeric events difficult, subapoptotic concentrations of the G4 ligands (>90% survival) were applied on A549 cells for long-term exposure. Population doubling was normal for up to a 45-day exposure for **12459** at 0.04 $\mu$M (Fig. 3*a*), whereas treatment with **115405** (0.4 $\mu$M) induced a 25–30% decline in population doubling up to 40 days exposure, without affecting cell viability, suggesting an increase in cell-cycle length. Prolonged exposure with either compound resulted in a population doubling plateau occurring at 40–45 days for both compounds, which ultimately led to cell division arrest at 70 and 53 days for **115405** and **12459**, respectively (Fig. 3*a*) and that corresponded to the failure of treated cells to replate. A 10-fold difference in drug concentration required to achieve growth arrest is observed between **12549** and **115405**. This observation may be due to differences in drug permeation, because A549 express slight amounts of MRP-1. The growth arrest was initially reversible, as cells treated with **115405** recovered within one week if the treatment was stopped at day 60 (before the final division arrest occurring at day 70; data not shown). Morphologic examination of the cells at the plateau phase showed an increased proportion of flat and giant cells with phenotypic characteristics of senescence and overexpression of $\beta$-galactosidase activity, (ref. 26; Fig. 3*d*). In addition, the proportion of apoptotic cells increased to 15–20% of the cell population, as compared with 0–4% for untreated controls. TRF analysis indicated a significant and progressive decrease of mean telomere length (Fig. 4*a Left*) that corresponds, approximately, to 1 kb or more for either **12459** or **115405**. Southern blot analysis also revealed the presence of some really short TRF fragments (<4 kb) in treated cells after day 42. Because TRF fragments also include subtelomeric regions, actual telomere length of treated cells is even shorter. Telomere erosion was pronounced during the initial drug treatment period and then stopped after $\approx$35 population doublings reaching a steady-state level of a mean TRF below 5 kb. Thus, this TRF size seems to be the minimal or critical mean length necessary to maintain cell division in the A549 cell line. When treatment with **115405** was stopped at day 60, TRF rapidly recovered a normal length, consistent with the arrest of telomerase inhibition in the cells and corresponding growth recovery (Fig. 4*a Right*). A slight overexpression of telomerase activity (1.5–2-fold) was measured by TRAP during TRF and growth recovery (not shown). The measure of TRF gives a global figure for the events occurring in the cells but minimizes specific events that occur in individual cells and

**Table 1. Cell proliferation inhibition by Triazines on a panel of human cell lines**

| Cell line | Type (tissue origin) | Telomerase activity | $IC_{50}$, $\mu$M | | | |
|---|---|---|---|---|---|---|
| | | | **115405** | **12459** | **5271** | **5332** |
| KB | Tumor (mouth) | **Positive** | 0.072 | >22.7 | n.d. | n.d. |
| BT20 | Tumor (breast) | **Positive** | 0.2 | >22.7 | n.d. | n.d. |
| HCT-116 | Tumor (colon) | **Positive** | 0.2 | >22.7 | n.d. | n.d. |
| SW-620 | Tumor (colon) | **Positive** | 0.32 | >22.7 | n.d. | n.d. |
| C8161 | Tumor (melanoma) | **Positive** | 0.6 | >22.7 | n.d. | n.d. |
| A549 | Tumor (lung) | **Positive** | 0.72 | 1.18 | 19.8 | >20.2 |
| LoVo | Tumor (colon) | **Positive** | 1.04 | 13.8 | n.d. | n.d. |
| HCT-8 | Tumor (colon) | **Positive** | 0.94 | >22.7 | n.d. | n.d. |
| HT29 | Tumor (colon) | **Positive** | 0.83 | >22.7 | n.d. | n.d. |
| U87 | Tumor (glioma) | **Positive** | 1.09 | 22.7 | n.d. | n.d. |
| A431 | Tumor (epidermis) | **Positive** | 1.8 | 6.5 | n.d. | n.d. |
| SKMEL-1 | Tumor (melanoma) | **Positive** | 2.04 | 3.1 | n.d. | n.d. |
| GM847DM | SV40 immortal. Fibroblast (skin) | **Negative, ALT*** | 1.2 | >10 | n.d. | n.d. |
| MRC5-Vi | SV40 immortal. Fibroblast (lung) | **Negative** | 0.12† | n.d. | n.d. | n.d. |
| hTERT-BJ1 | Telom. immortal. Fibroblast (foreskin) | **Positive** | 0.9 | 1.1 | >20 | n.d. |

n.d., Not determined.
*ALT, alternative lengthening of telomere.
†Measured by clonogenic survival.

chromosomes. Analysis of telomere distribution with a fluorescent telomere probe (37) [fluorescence *in situ* hybridization (FISH)] was used to validate these results. Although the karyotype of A549 cells (60 chromosomes at metaphase) is quite different from normal cells, it was possible to distinguish significant differences in telomere labeling between **115405**-treated and untreated cells at 35 population doublings (Fig. 4*b*). Chromosomes with four distinguishable fluorescent telomeric spots completely disappeared, whereas chromosomes without any telomeric staining increased from 9% to 25% (Fig. 4*c*). Another indication of the telomeric target of these agents was obtained through an analysis of the effect of **12459** and **115405** (0.1 $\mu$M) during the anaphase of A549 cells. Compounds induced figures of anaphase-bridging (not shown) as already reported for TMPy4, another G4-interacting agent, in sea urchin embryos (38).

Another telomerase-positive cell line with shorter telomeres was tested to further confirm that this growth arrest could be correlated with a decrease in telomere length. Treatment of A431 cells with a low, subapoptotic concentration of **115405** (0.2 $\mu$M) also induced a delayed growth arrest. This arrest was observed after a shorter lag time as compared with A549 cells: only 15 days of continuous treatment were required to obtain a plateau where apoptosis was the major hallmark of growth arrest (Fig. 3*b*). Similar results were obtained by using dominant negatives of hTERT on this cell line (39). This cell line bears telomeres with an initial TRF length of $\approx$4 kb (39), suggesting that the duration of treatment needed to achieve senescence induction with G4 ligands may be directly related to the initial length of the telomere, as already demonstrated for antisense oligonucleotides (40, 41).

Long-term treatment of a normal human fibroblast cell line immortalized by hTERT [hTERT-BJ1 (26)] that exhibits a considerable increase in its telomere length (>10 kb) was undertaken in the presence of active triazines (**115405** and **12459**) and an inactive one (**5271**). Results presented in Fig. 3*c* indicated that **5271** had no effect on the long-term growth of hTERT-BJ1 cells, as compared with untreated cells. **115405**, but not **12459**, slightly affected the cell growth as already observed for A549 cells (see also Fig. 3*a*), but without any indication of a plateau after 100 days. However, the appearance of a few senescent cells, as evidenced by $\beta$-galactosidase activity, started after 55 days of treatment. Such results indicate that long-term effects of G-quadruplex interacting agents might take significantly longer time to achieve replicative senescence on normal cells where telomeres were increased in size, as compared with tumor cells. This difference might represent the ''therapeutic index'' necessary for a treatment using these agents.

In summary, two triazine compounds have been shown to increase the melting temperature of a telomeric quadruplex and appear to be among the most potent nonnucleoside telomerase inhibitors reported to date. Trisubstituted acridines are also potent G-quadruplex ligands and telomerase inhibitors, although no cellular evidence for telomere shortening with these molecules is currently available (42). A catalytic inhibitor of telomerase, BIBR 1532, was reported to induce reversible telomere shortening in cancer cells (43). Our work provides evidence that G-quadruplex ligands are also able to induce telomerase shortening in cancer cells. In this triazine series, a good correlation is found between telomerase inhibition and quadruplex affinity. Specific telomerase inhibition is compatible with our knowledge of the binding mode of these ligands with G-quadruplex DNA. The relationship between structure, activity, and specificity will need further structural analysis to be understood. One should also keep in mind that short-term activity may result from selective effects on G-quadruplexes or nonselective interactions with other forms of nucleic acids. Although recent publications have demonstrated that minor changes in the RNA component of telomerase (hTR) or its protein component (hTERT) induced dramatic and rapid consequences on cell viability (35, 36), a direct link between short-term apoptosis and interaction with G-quadruplexes remains to be demonstrated. The recurrent question of the existence of G-quadruplexes in eukaryotic cells was recently elucidated in ciliates. Specific antibodies to G-quadruplexes demonstrated the presence of these DNA structures at *Stylonychia lemnae* telomeres (44). These data argue in favor of a direct interaction of triazines with G-quadruplexes to block telomerase activity in mammalian cells. G-quadruplex DNA structures are potentially distributed at various other sites of the human genome, such as gene promoters and rDNA and these sites may represent additional targets involved in the mechanism of action of triazines. Stabilization of G-quadruplex at one or several sites of the genome may impair DNA replication and/or transcription. G4

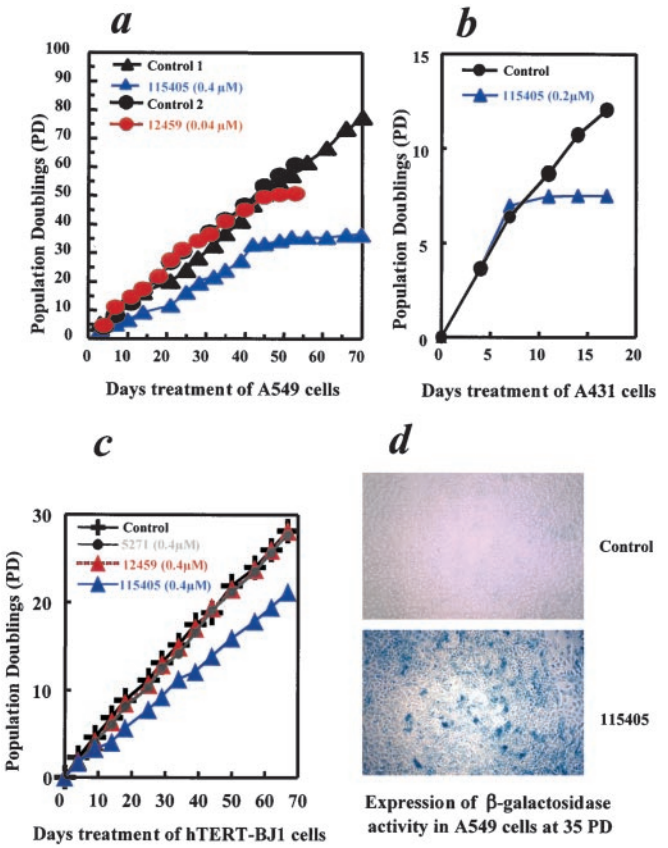


**Fig. 3.** Delayed growth arrest and senescence-like phenotype induced by long-term treatment. (*a*) Cells with medium telomere length (6 kb). A549 cells were maintained in culture in the presence of **12459** (0.04 $\mu$M) or **115405** (0.4 $\mu$M) for an extended period. A cell-growth plateau appears at day 45, compared with control untreated cells. Cultures could not be replated at day 53 for **12459** and day 70 for **115405**. Growth arrest corresponds to the appearance of a senescence-like phenotype and an increase in the number of apoptotic cells. (*B*) Cells with short telomere length (4 kb). A431 cells were maintained in culture in the presence of **115405** (0.2 $\mu$M) for an extended period. Culture could not be replated at day 66. Growth arrest corresponds to an increase in the number of apoptotic cells. (*C*) Cells with long telomere length (>10 kb). hTERT-BJ1 cells were maintained in culture in the presence of **5271** (0.4 $\mu$M), **12459** (0.4 $\mu$M), or **115405** (0.4 $\mu$M) for up to 70 days. Cells were still dividing at day 100 (not shown) and the appearance of cells expressing $\beta$-galactosidase activity started at day 55 only for **115405**. (*d*) Expression of $\beta$-galactosidase activity in A549 cells treated (*Lower*) or untreated (*Upper*) with **115405** (0.4 $\mu$M) harvested at 35 population doublings (35 PD). Treatment with **115405** increases the number of senescent-like cells (*Lower*).

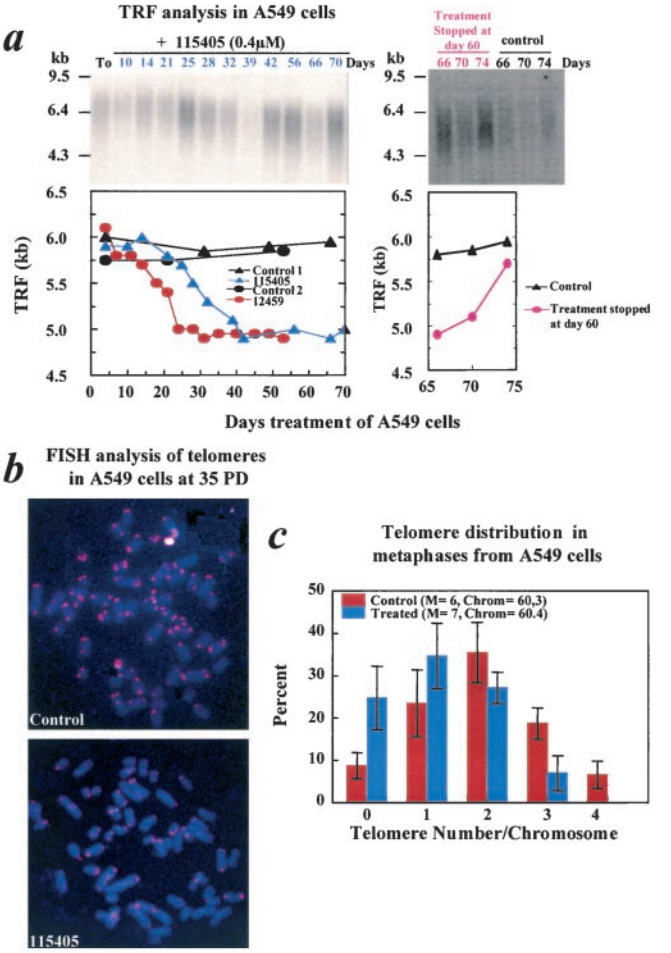


**Fig. 4.** Effect of triazines on telomere length. (*a Left*) TRF analysis in A549 cells treated with **115405** (0.4 $\mu$M) and harvested at different times (days) of the culture. "$T_o$" corresponds to control-untreated A549 cells harvested at day 32 (*Upper*). TRF mean values (kb) were expressed as a function of the duration of treatment (days) for **115405** and **12459** (*Lower*). (*a Right*) TRF analysis in A549 cells pretreated for 60 days with **115405** (0.4 $\mu$M), then untreated and harvested at days 66, 70, and 74. Control-untreated cells seeded at day 0 were harvested at days 66, 70, and 74 (*Upper*). TRF values (kb) were expressed as a function of the duration of the treatment for control or untreated at day 60 cells (*Lower*). (*b*) Representative images of *in situ* hybridization of Cy3-telomeric probe (red) to metaphase chromosome (blue). Fluorescence *in situ* hybridization (FISH) analysis of telomeric repeats in A549 cells harvested at 35 population doublings, using a fluorescent oligonucleotide (31). (*c*) Analysis of *in situ* hybridization in metaphases from control or **115405**-treated A549 cells. Red and blue bars indicate the percentage of the mean number of telomeres/chromosome ($\pm$ SD) calculated in six metaphases (control) and seven metaphases (treated). Chrom is the mean number of chromosomes/metaphase.

ligands induced down-regulation of the *c-myc* gene expression, a gene which contains a G-quadruplex-forming sequence in its promoter, and were found to be potent inhibitors of the G-quadruplex-specific helicases from the RecQ family (45–48). The demonstration that other G-quadruplexes might be implicated in short- or long-terms effects of triazines or other related ligands still remains to be elucidated. Preliminary experiments suggest that **12459** and **115405** do not distinguish between the different classes of G-quadruplexes. The activity of **115405** on telomerase-negative ALT (Alternative Lengthening of Telomere) cells also indicates a major difference between an inhibitor of telomerase activity and a G-quadruplex interacting agent. Although G4-ligands may act as potent telomerase inhibitors, their main property is to stabilize G-quadruplexes. ALT involves recombination processes and the participation of G-quadruplex helicases is strongly suspected (48, 49). Therefore, it will be of interest to determine the action of triazines on telomeric DNA structures and against helicases in such cells. To further investigate potential additional mechanisms of action of this series, mutagenesis experiments to obtain resistance toward triazines have been initiated. Compound **115405** has been selected as a new antitelomerase agent for further preclinical studies. Its novel long-term cellular properties can be exploited to induce senescence at protracted low dosage, especially in the case of tumors with relatively short telomeres.

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