Resistance to Senescence Induction and Telomere Shortening by a G-Quadruplex Ligand Inhibitor of Telomerase

Dennis Gomez, Nassera Aouali, Alexandre Renaud, Céline Douarre, Kazuo Shin-ya, Jamal Tazi, Sophie Martinez, Chantal Trentesaux, Hamid Morjani, and Jean-François Riou

Onco-Pharmacologie [D.G., C.D., C.T., J-F.R.J., Unité Mixte de Recherche (UMR) 6142 Centre National de la Recherche Scientifique (CNRS) [N.A., A.R., C.D., C.T., H.M.J., and Equipe d’Accueil 2070 [S.M.]], Institut Fédéré[1] de Recherche 53, Unité de Formation et de Recherche de Pharmacie, Université Reims Champagne-Ardenne, 51096 Reims Cedex, France; Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan; and Institut de Génétique Moléculaire, UMR 5535 CNRS, IFR 122, 34293 Montpellier cedex, France [J.T.]

Abstract

The molecular mechanisms induced by G-quadruplex ligands to trigger senescence in mammalian cells are still unknown, although the critical role of telomerase is highly suspected. JFA2 cells selected for resistance to senescence induced by the G-quadruplex ligand 12459 presented an overexpression of hTERT transcript that correlated to a functional increase in telomerase activity and telomere length. Consistently, treatment with 12459 failed to trigger senescence and telomere shortening in JFA2 cells. Resistant cells also presented cross-resistance for senescence induction to telomestatin, another G-quadruplex ligand from a different series, but not to other anticancer agents, indicating the selectivity of the resistance mechanism. We, thus, provide evidence that telomerase activity and telomere length are key cellular determinants of the resistance to G-quadruplex ligands.

Introduction

Telomeres are essential to maintain the stability of chromosomal ends, and telomeric overhang has been implicated as a critical component of telomere structure that is required for proper telomere function (1, 2). Telomeric overhang may be involved in different DNA conformations such as T-loops (2) or G-quadruplexes (3). The presence of telomeric quadruplexes has been recently demonstrated in the macronucleus of a ciliate, Stylonychia lemnana (4). G-quadruplexes were also present in other parts of the genome such as gene promoters (5), and it was proposed that their formation must be strictly controlled during replication to avoid genetic instability (6).

Telomere replication is sustained 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 expressed in most somatic cells (7). Such differential expression provided the initial rationale for the evaluation of telomerase inhibitors as potential anticancer agents. Folding of the telomeric G-rich overhang into a quadruplex DNA has been found to inhibit telomerase activity. Therefore, stabilization of the telomeric overhang into a G-quadruplex structure by specific ligands can then be an original strategy to achieve antitumour activity (8–10). The G-quadruplex, which is very different from classical double-stranded B DNA, provides a good structural basis for selective recognition, and several classes of small molecules that selectively bind to G-quadruplex DNA and inhibit telomerase activity have been described (8–10).

The 2,4,6-triamino-1,3,5-triazine derivatives are potent telomerase inhibitors that bind to telomeric G-quadruplex (11). In this series, 12459 is one of the most selective G-quadruplex-interacting compounds that displayed a 25-fold selectivity when telomerase inhibition was compared with the Taq polymerase inhibition by using the TRAP-G4 assay (12). In addition, 12459 is able to induce both telomere shortening and senescence in the human lung adenocarcinoma A549 cell line, as a function of its concentration and time exposure (11). The molecular mechanism induced by G-quadruplex ligands to trigger senescence in mammalian cells is still unknown, although the critical role of telomerase is highly suspected.

In the present study, we used the strategy to render tumor cells resistant to the senescence induced by a G-quadruplex ligand to analyze cellular determinants of the resistance. We show that resistant cells presented a functional alteration of telomerase activity and telomere length that lead to a specific resistance to senescence and telomere shortening triggered by G-quadruplex ligands.

Materials and Methods

Oligonucleotides and Compounds. All oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). The triazine derivative 12459 was synthesized according to patent WO-0140218. Telomestatin was purified as described previously (13). Other compounds were commercially available (Sigma Chemical Co.). Solutions of compounds were prepared in 10 mM DMSO, except telomestatin, which was prepared in 5 mM methanol/DMSO (50:50). Additional dilutions were made in water.

Telomerase Assay. Telomerase extracts were prepared from A549 and JFA2 cells as described previously (11). The TRAP assay was performed in the presence of an internal control (internal telomerase assay standard) corresponding to the 36-mer (5’TCCCTCTGAGGAGGTTAAGGC-CGAGAAGCGAT-3’) described previously (14).

Cell Culture Conditions and Selection of Resistant Cell Lines. The A549 human lung carcinoma cell line was from American Type Culture Collection. These cells were grown in DMEM with Glutamax (Invitrogen), supplemented with 10% FCS and antibiotics.

For long-term growth of A549 cells, treated or untreated cells were seeded at 6 × 10^4 cells into 25-cm² tissue culture flask for 4 days, then trypsinized and counted. Each time, 6 × 10^4 cells were replated onto a new culture flask with fresh drug solution. The JFA-resistant cell line was established by progressive adaptation to long-term treatment with 12459. The starting concentration was 0.05 μM and was progressively increased to 0.1, 0.15, 0.2, and 0.3 μM. Fresh drug solution was added at each passage. The JFA2 cell line is derived from JFA 0.2 that was further grown in the absence of 12459. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide survival assay (4 days), in the presence of various cytotoxic compounds, was performed in 96-well plates, each point in quadruplicate, as recommended by the manufacturer.

Received 5/14/03; revised 7/10/03; accepted 8/5/03.

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1 Supported by an Action Concertée Incitative “Molécules et Cibles Thérapeutiques” grant from the French Ministry of Research and a grant from the Association pour la Recherche sur le Cancer (Grant 4691).

2 To whom requests for reprints should be addressed, at Unité de Formation et de Recherche de Pharmacie, Université Reims Champagne-Ardenne, 51 rue Cognacq-Jay, 51096 Reims Cedex, France. Phone: 33-3-26-91-80-13; Fax: 33-3-26-91-37-30; E-mail: jf.riou@univ-reims.fr.

3 The abbreviations used are: TRAP, telomeric repeat amplification protocol; TBE, Tris-borate EDTA; PD, population doubling; TRF, telomere restriction fragment.
manufacturer (Sigma Chemical Co.). The resistance index was the ratio of the drug IC_{50} for JFA2 over A549 and corresponded to the mean value (+/− SD) of three independent experiments.

RNA Preparation and RT-PCR Assays. Total RNA was isolated from 1 × 10^6 cells using Tri-Reagent (Sigma Chemical Co) as recommended by the manufacturer. One microgram 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 diethyl pyrocarbonate-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'-GCCCTGACGTGTACCTTGTCAC-3') and the reverse TERT2531R primer (5'-GGCTGAACAGCTGAGAGGAGG-3') as described previously (15), 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'-CCCCCACTGAAAAAGATTGA-3') and primer reverse (5'-ATCTTTAAACCTCCATGATG-3'). Amplified products were resolved on 6% nondenaturing polyacrylamide gels in 1× TBE and stained with SYBR Green I (Roche). Quantification was performed by a charge-coupled device camera (Biochip) and a BioCapt software analysis, relative to the signal of β2-microglobulin. Results represent the mean value of three independent RNA extractions.

Telomeric Restriction Size Fragment Determination. Genomic DNA was digested with HindIII/RsaI restriction enzymes and electrophoresed on 0.8% agarose gels in 1× TBE buffer. DNA were transferred onto nitrocellulose membrane (Amersham) and then prehybridized for 2 h at 65°C in 7% SDS, 1% BSA, and 0.5 mM NaPO4 (pH 8.0). A 0.7-kb telomere DNA probe (pUCTelo; a gift from Prof. E. Gilson, École Normale Supérieure, Lyon, France) was labeled with [32P]dCTP by random-priming and was added to the solution. Hybridization was performed overnight at 65°C. Membranes were washed twice with 0.2× SSC and 0.1% SDS at 65°C for 15 min, then twice with 2× SSC and 0.1% SDS at room temperature. Telomeric smears were revealed by exposure in a phosphorimagery (Typhoon 9210; Amersham).

Solution Hybridization Experiments. The nondenaturing hybridization assay to detect 3’ telomere overhang was performed with a modification of the procedure described previously (16). Aliquots of 5 μg of undigested genomic DNA were hybridized at 57°C overnight with 0.5 pmol of [γ-32P]ATP-labeled (CCCTAA)_n oligonucleotide in hybridization buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl2, and 1 mM DTT) in the presence or absence of RsaI and HindIII restriction enzymes in a volume of 20 μl. Reaction was stopped with 2 μl of 1% SDS and 1 mg/ml Proteinase K and incubated for 30 min at 50°C. Hybridized samples were size-fractionated on 0.8% agarose gels in 1× TBE buffer. The gels were stained with ethidium bromide, washed, and dried on Whatman filter paper. Ethidium fluorescence and radioactivity were scanned in a phosphorimagery (Typhoon 9210; Amersham). The procedure allows detecting the amount of single-strand overhang available for hybridization (undigested sample), and migration sizes represent overall telomere length (RsaI and HindIII digested sample).

Results and Discussion

We have investigated the cellular consequences of the induction of resistance to the triazine derivative 12459, using the appearance of senescence as a selection criterion. Derivative 12459 was chosen inside the triazine series because of its high selectivity profile (12). A549 cells were treated with a 12459 starting concentration equal to 0.04 μM, which was progressively increased to 0.1, 0.15, 0.2, and 0.3 μM. Parallel treatment of A549 cells with a fixed 12459 concentration equal to 0.04 μM induced a plateau after 40 days on growth curves, followed rapidly by an arrest in cell growth at day 60 with typical figures of senescent cells (Fig. 1A). Cells treated with the progressive increase of 12459 concentrations up to 0.3 μM (Fig. 1B, JFA 0.3) entered a plateau at day 85, followed by an arrest of cell growth at day 95 (approximately PD 70). In contrast, cells treated up to 0.2 μM (JFA 0.2) were able to continue growth up to 225 days (Fig. 1B and data not shown) and, therefore, became resistant to the drug treatment. The growth curve of JFA 0.2 presented a slight decrease in proliferation between days 80 and 90 (approximately PD 80) that disappeared later. We suspected that cells passed through an event(s) that may modify their sensitivity to additional drug treatment. To elucidate that point, 12459 concentration was increased to 0.3 μM on JFA 0.2 at PD 90 and was pursued up to PD 180 without any evidence of senescence (data not shown). Altogether, these results indicated that JFA 0.2 cells have become 5- to 7.5-fold resistant to 12459 after PD 80, as compared with A549 parental cells. Although JFA 0.2 cells never entered senescence, an apparent delay on cell growth was observed corresponding to an increase in doubling time to 27.5 h, as compared with untreated A549 cells (20-h doubling time). The doubling-time modification is mainly dependent on the 12459 treatment because its removal at day 110 (PD 90) restored a growth nearly similar to that of untreated A549 cells with a doubling time equal to 22 h. Additional evaluations were performed on untreated JFA 0.2, renamed JFA2.

JFA2 was evaluated for its cross-resistance pattern to other cytotoxic agents with various mechanisms of action. As shown in Fig. 1C, JFA2 has no cross-resistance to the topoisomerase inhibitors doxorubicin, etoposide, and camptothecin; to the DNA-interactive agent mitomycin C; or to the tubulin poison vinblastin. Interestingly, a 3- to 4-fold collateral sensitivity was found for etoposide and vinblastin. This sensitivity may be related to vinblastin for an increased amount of mitotic alteration (anaphase bridges) observed in JFA2 that could enhance the effect of this mitotic poison (data not shown). Etoposide...
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Telomerase activity was found augmented in JFA2 cells, as compared with parental cells (Fig. 2C). Interestingly, treatment of A549 with 12459 (10 μM, 48 h) had no effect on the global transcription level of hTERT but markedly altered its splicing pattern, leading to a strong decrease of the active transcript (+α, +β) and to an increase of the inactive one. In sharp contrast, treatment of resistant JFA2 cells with 12459 did not alter the splicing pattern. The active (+α, +β) transcript was reproducibly maintained at a level comparable with that of untreated cells, and the inactive −β transcript was poorly modified. In agreement with these observations, telomerase activity measured by TRAP in A549-treated cells was down-regulated but remained detectable in JFA2-treated cells (with a level comparable with that of untreated A549 cells; Fig. 2C). Because short-term treatment with 12459 is able to down-regulate telomerase activity, we have also determined whether long-term treatment with a lower 12459 concentration might also modulate telomerase activity. A549 cells treated with 0.2 μM 12459 during the resistance acquisition at day 100 presented a strong decrease of telomerase activity measured by TRAP (Fig. 2D). In JFA2 cells, the arrest of 12459 treatment progressively restore telomerase activity that became up-regulated after four passages without 12459, as compared with parental A549 cells (Fig. 2D and data not shown). These data suggested that the relaxation of the 12459 pressure might be at the origin of the hTERT and telomerase activity overexpression in JFA2. The in vitro inhibitory effect of 12459 was also measured by the TRAP assay on extracts from sensitive and resistant cells. Derivative 12459 was found to inhibit TRAP with equal IC50 for sensitive and resistant extracts. This result excludes a qualitative alteration of telomerase that modify the sensitivity of the enzyme to the inhibition by 12459 (data not shown). These data suggested that overexpression of telomerase activity and a failure of 12459 to reduce the expression levels of hTERT, through alternative splicing, in JFA2 cells are the characteristics of the resistance. Furthermore, we have obtained in the laboratory clones of another 12459-resistant cell line selected by mutagenesis. These resistant clones presented a 2- to 5-fold hTERT overexpression, increased telomerase activity, and telomere length.4 Therefore, up-regulation of telomerase expression seems to represent a frequent mechanism to escape from the cellular effects of 12459.

Telomestatin is another G-quadruplex ligand described as a potent and selective inhibitor of telomerase (13). To determine whether JFA2 cells are cross-resistant to the senescence induced by telomestatin, we have treated long-term cultures of A549 and JFA2 with different concentrations of this compound (0.5, 1, and 2 μM). Telomestatin at 0.5 μM does not induce senescence on both A549 and JFA2 cells after 60 days (Fig. 3, A and B). An increase of telomestatin to 1 μM induced a plateau at day 16 on A549 cells, followed by senescence at day 20 (Fig. 3A), in contrast to JFA2 cells that were still able to grow after 60 days of treatment (Fig. 3B). In the presence of 2 μM telomestatin, plateau and senescence of A549 cells were also achieved in A549 after 8 and 20 days, respectively (Fig. 3C). Telomestatin treatment was able to lower the growth of JFA2 cells up to 20 days, but then after, the cell line recovers a normal growth and does not enter senescence up to 30 days (Fig. 3D). As a control, A549 and JFA2 cell lines were also treated with 0.2 μM 12459. In that case, the senescence was obtained after 20 days of treatment on A549 cells but not on JFA2 cells (Fig. 3, C and D).

Altogether, these data indicated that JFA2 cells presented a resistance to senescence induction toward telomestatin. Resistance of JFA2 cells to 12459 was also achieved by using a single concentration of the

compound on a cell line grown without 12459, which indicated that the phenotype acquired during resistance selection is stable.

The latter observation prompted us to analyze the telomere length in A549 and JFA2 cells during treatment with telomestatin and 12459. JFA2 have larger TRF length (~12 kb) than the parental A549 cells (~7 kb; Fig. 4A, compare Lanes 1 and 3). We have also determined by non-denaturing solution hybridization analysis and native agarose gel electrophoresis the signal hybridizing at 3' telomere overhang. Comparison between hybridization signals and ethidium bromide staining for undigested A549 and JFA2 DNA samples do not indicate significant change in the 3' telomere overhang (Fig. 4B, compare Lanes 1 and 3). In agreement with the TRF experiment, RsaI- and Hinfl-digested DNA samples also revealed that the size of the overall telomere length hybridizing with the telomeric probe is increased in JFA2 as compared with A549 cells (Fig. 4B, compare Lanes 2 and 4). The homogenous long size of JFA2 telomeres are in favor of a functional overexpression of telomerase in resistant cells. Because the JFA2 cell line was grown without 12459 for 120 days to obtain a 5-kb net gain of telomeres, it corresponded to a 40-base increase per PD. In normal cells without telomerase, telomeres erode from 50 to 200 bases at each round of division (7). Therefore, our results are consistent with the 2-fold activation of the hTERT transcript, although it could not be excluded that resistance contributed to the selection of cells containing long telomeres through an unknown mechanism mediated by 12459.

Long-term treatment of A549 cells with 12459 induced a telomere shortening as reported previously (11). Similarly, telomestatin at 0.1 μM triggered significant telomere shortening against A549 cells (Fig. 4A, Lane 2). In contrast, DNA samples from JFA2 cells treated with 0.1 μM telomestatin did not show significant change in TRF length, as compared with untreated JFA2 (Fig. 4A, compare Lanes 3 and 4). Similar results were obtained with JFA2 cells treated with 0.5 or 2 μM telomestatin or 0.2 μM 12459 (results not shown).

These results indicate that JFA2 cells are also resistant to telomere shortening induced with either 12459 or telomestatin, thus suggesting that resistance to senescence induction is linked with resistance to telomere shortening, in good agreement with its increased telomerase activity.

Interestingly, long-term treatment of both sensitive and resistant cells with 12459 and telomestatin are able to induce a growth inhibition, without affecting cell viability, which is uncoupled from the senescence induction and the telomere shortening in the resistant cells. It may be proposed that despite the important selectivity of these G-quadruplex-interacting agents, additional mechanisms unrelated to telomere shortening and telomerase inhibition may be involved in this growth inhibition process (9, 10). It is noteworthy that other potential quadruplexes are located throughout the genome (19, 20) and a quadruplex in the c-myc gene was recently highlighted to modulate c-myc expression and cell growth (5).

Interestingly, the senescence and telomere shortening induced by 12459 and telomestatin against the parental cells arise earlier than expected for a catalytic inhibition of telomerase but too late to be the consequence of an unrelated cytotoxic event. In agreement, the expression of a hTERT dominant negative cDNA in A549 cells, inhibiting catalytic activity of telomerase, induced a progressive telomere shortening as reported previously (11). Similarly, telomestatin at 0.1 μM triggered significant telomere shortening against A549 cells (Fig. 4A, Lane 2). In contrast, DNA samples from JFA2 cells treated with 0.1 μM telomestatin did not show significant change in TRF length, as compared with untreated JFA2 (Fig. 4A, compare Lanes 3 and 4). Similar results were obtained with JFA2 cells treated with 0.5 or 2 μM telomestatin or 0.2 μM 12459 (results not shown).

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shortening. Extensive and irreversible degradation of telomeres was recently observed during the late phase of apoptosis (21) but may not correspond to the present situation, because 12459 was not found to induce significant apoptosis at the concentrations used in these experiments and that telomere attrition induced by 12459 was found reversible (11). Furthermore, the G-quadruplex ligand concentrations used to trigger senescence are largely below those necessary to induce short-term (96 h) antiproliferative activity in A549 (IC₅₀ 12459, 1.8 μM; IC₅₀ telomestatin, 9.6 μM). These concentrations are rather compatible with a mechanism related to telomerase inhibition (ranging from 0.15 to 0.6 μM) than to an unrelated cytotoxic event, because TRAP inhibition was found between 0.1 and 0.6 μM for these ligands (11, 12). It should also be noted that the molecular target of these ligands is DNA itself (i.e., the 3' overhang of telomere). Therefore, telomerase that catalyzes the overhang extension has to be considered as an indirect target of these ligands. Because telomerase displays both catalytic and telomere capping functions (22), it is possible that telomere shortening and subsequent senescence induced by G-quadruplex ligands might also result from telomere uncapping that trigger more dramatic and rapid consequences on cell viability than a mechanism of telomere attrition. Therefore, the mechanisms related to the senescence induced by G-quadruplex ligands might be a subtle balance between telomerase inhibition and telomere uncapping, as a function of the ligand concentration.

Interestingly, our results indicated that JFA2 cells presented resistance to all putative mechanisms by which G-quadruplex ligands might induce telomere shortening. It could be also speculated that telomere lengthening in resistant cells may be caused by a telomerase-independent mechanism selected during the resistance acquisition. In that case, the expression of a DN-hTERT would not be expected to induce telomere shortening in the resistant cells. Such an experiment was done on a 12459-resistant clone (JFD18) that presented a similar resistance-associated phenotype (hTERT overexpression and increased telomere length; cell line to be described elsewhere). In conclusion, our results are the first evidence that cellular resistance to a G-quadruplex ligand is related to phenotypic alterations that are selective to G-quadruplex ligands and not to other anticancer agents. Previous studies have suggested a link between senescence induction and telomere shortening induced by these inhibitors. We believe that up-regulation of telomerase activity, lack of telomere shortening, and cross-resistance with telomestatin for senescence induction in JFA2-resistant cells reflect a situation in which telomere and telomerase are important targets for the molecular action of these ligands in cancer cells.

**Acknowledgments**

We thank J. L. Mergny, P. Mailliet, E. Mandine, L. Lacroix, and A. Londono-Vallejo for helpful discussions and constant support and D. Pisani for technical help.

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