

Biological Activity of the G-Quadruplex Ligand RHPS4 (3,11-Difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*k*]acridinium methosulfate) Is Associated with Telomere Capping Alteration

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Received April 16, 2004; accepted August 3, 2004

ABSTRACT

This study had two goals: 1) to evaluate the biological effect of the novel pentacyclic acridine 3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*k*]acridinium methosulfate (RHPS4) on human melanoma lines possessing long telomeres, and 2) to elucidate the relationship between G-quadruplex-based telomerase inhibitor-induced cellular effects and telomere length/dysfunction. The cellular pharmacological effects of RHPS4 have been evaluated by treating melanoma lines with increasing concentrations of RHPS4. A dose-dependent inhibition of cell proliferation was observed in all the lines during short-term treatment. Flow cytometric analysis demonstrated that RHPS4 induced a dose-dependent accumulation of cells in the S-G₂/M phase of

cell cycle. The RHPS4-induced cell cycle alteration was irreversible even at low doses, and the cells died from apoptosis. At high RHPS4 concentration, apoptosis was accompanied by the induction of a senescence phenotype: large cell size, vacuolated cytoplasm, and β -galactosidase activity. The short-term biological activity of RHPS4 was not caused by telomere shortening, but it was associated with telomere dysfunction, in terms of presence of telomeric fusions, polynucleated cells, and typical images of telophase bridge. In conclusion, our results demonstrate that the G-quadruplex ligand RHPS4 can function in a telomere length-independent manner through its ability to cause telomere-capping alteration.

Telomerase is a complex ribonucleoprotein reverse transcriptase responsible for telomere protection and maintenance (Morin, 1989), and its expression is associated with cell immortalization and tumorigenesis (Bodnar et al., 1998). Telomerase is overexpressed in the majority of human tumors, whereas it is not detected in most somatic cells (Shay and Bacchetti, 1997). Such differential expression was the initial rationale for the evaluation of telomerase inhibitors as potential anticancer drugs (Sharma et al., 1997). Several classes of telomerase/telomere machinery inhibitors were evaluated: dominant-negative hTERT protein (Hahn et al., 1999), oligonucleotides and ribozymes targeting human tem-

plate RNA or hTERT mRNA (Pitts and Corey, 1998; Mukai et al., 2000), PNA molecules (Norton et al., 1996), and small-molecule telomerase inhibitors (Naasani et al., 1999; Damm et al., 2001). In some of these cases, inhibition of telomerase in various cell lines resulted in cellular senescence or apoptosis in a time-dependent manner that correlated with the initial telomere length (Hahn et al., 1999). The presumed long lag period required before observing telomere attrition, and consequently cellular growth arrest or apoptosis, is one of the major limitations for the development of clinically useful telomerase inhibitors. As an alternative, tumor cell crisis can be provoked rapidly by inducing telomere dysfunction. The strategy of direct targeting of telomeres, rather than the telomerase enzyme, may cause effects on telomere capping independent of the initial telomere length. Recent evidence suggests that uncapped telomeres can suffer degradation, inappropriate recombination, and end-to end fusions

This work was supported by grants from Associazione Italiana Ricerca sul Cancro, Consiglio Nazionale delle Ricerche, and Ministero della Salute and Ministero dell'Istruzione, dell'Università e della Ricerca.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.104.001537.

ABBREVIATIONS: hTERT, human telomerase reverse transcriptase; PNA, peptide nucleic acid; TUNEL, terminal deoxynucleotide transferase [TdT]-mediated dUTP nick-end labeling; TRAP, telomeric repeat amplification protocol; SA- β -gal, senescence- β -galactosidase; ALT, alternative lengthening of telomeres.

manifested as anaphase bridge (Blackburn, 2000; van Steensel et al., 1998) and that telomere dysfunction can have an effect similar to that of DNA damage in eliciting cell cycle arrest or apoptosis (Karlseder et al., 1999).

Molecules capable of interacting with and stabilizing four-stranded G-quadruplex structures formed by the G-rich single-stranded overhang of telomeres have been generated (Sun et al., 1997). These agents are indirect telomerase inhibitors capable of stabilizing the G-quadruplex structure and interfering with telomere replication by blocking the elongation step catalyzed by telomerase (Zahler et al., 1991). Classes of G-quadruplex inhibitors described so far include anthraquinones (Sun et al., 1997; Perry et al., 1998), fluorenones (Perry et al., 1999b), acridines (Harrison et al., 1999), cationic porphyrins (Shi et al., 2001; Izicka et al., 1999), a perylenetetracarboxylic diimide derivative (Fedoroff et al., 1998), indoloquinolines (Caprio et al., 2000), and a benzonaphthofurandione tetracyclic compound (Perry et al., 1999a).

A challenge for the development of G-quadruplex-interacting molecules is the relatively poor selectivity for binding to quadruplex versus duplex DNA, causing short-term cell kill. The novel pentacyclic acridine RHPS4 (Fig. 1) has a high selectivity for quadruplex DNA structure (Gowan et al., 2001; Heald et al., 2002; Gavathiotis et al., 2003). RHPS4 inhibits telomerase at submicromolar levels and exhibits a wide differential between telomerase inhibition and short-term cytotoxicity. When RHPS4 was added to 21NT and A431 human tumor cells, which have relatively short telomeres, a marked cessation in cell growth was observed. On the contrary, no growth-inhibitory effects were observed in SKOV-3 cells, which possess relatively longer telomeres. However, the hypothesis that RHPS4 could work only in a telomere length-dependent manner was questioned by the demonstration that RHPS4 also conferred a growth-inhibitory effect against GM847 ALT cells in a short time, even though these cells possess very long telomeres (Gowan et al., 2001).

The goal of this study was to evaluate the cellular pharmacological effects of RHPS4 on other tumor lines that possess relatively long telomeres and to elucidate the relationships between G-quadruplex-based telomerase inhibitor-induced cellular effects and telomere length/dysfunction. To this aim, on the basis of our previous data demonstrating that telomere maintenance by telomerase plays a crucial role in melanoma tumorigenicity and drug sensitivity (Biroccio et al., 2002, 2003a,b), a panel of human melanoma lines, previously established in our laboratory, was used.

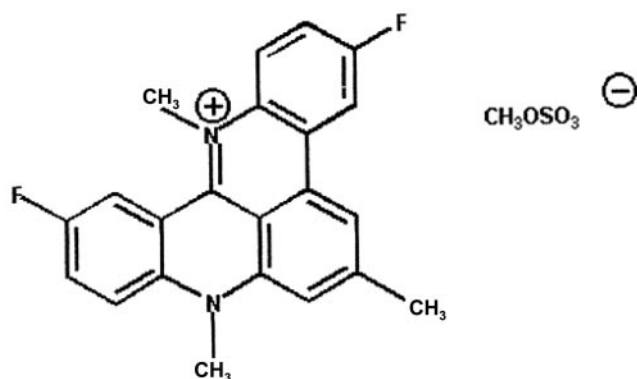


Fig. 1. Chemical structure of the pentacyclic acridine RHPS4

Materials and Methods

Cells and Culture Conditions. Six human melanoma lines (M14, PLF2, JR1, JR8, SBCL1, and SAN) were maintained as monolayer cultures in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics at 37°C in a 5% CO₂-95% air atmosphere. Except for the M14 line, the other lines were obtained from patients biopsy at the Surgery Department of Regina Elena Cancer Institute (Roma, Italy).

Proliferation Assays. For short-term assay, 5×10^4 cells were seeded in 60-mm Petri plates (Nalge Nunc International, Naperville, IL); 24 h after plating, increasing concentrations of freshly dissolved RHPS4, ranging from 0.2 to 10 μ M, were added to the culture medium and left for 4 days. Then, at day 5, the drug was readded to medium and left for an additional 4 days. Cell counts (Beckman Coulter, Fullerton, CA) and viability (trypan blue dye exclusion) were determined daily, from day 1 to day 10 of culture. The RHPS4 dose inhibiting cell proliferation by 50% (IC₅₀) was calculated at days 5 and 7 of treatment.

For long-term assay, cells were exposed to 0.2 and 0.5 μ M RHPS4 for 4 days. Then, the number of adherent cells in the dish was counted, and the cells were replated and treated again. This procedure was repeated for 10 in vitro passages (50 days). The increase in population doubling level was calculated according to the formula (Δ PDL = $\log(n_t/n_0)/\log 2$, where n_0 is the initial number of cells and n_t is the final number of cells).

Flow Cytometric Analysis. Cell cycle analysis was performed by flow cytometry (BD Biosciences, Heidelberg, Germany). Adherent cells (2×10^5) were fixed and resuspended in a solution containing propidium iodide at a concentration of 50 μ g/ml. Cell percentages in the different phases of the cell cycle were measured using CELLQuest software (BD Biosciences).

Morphological Analyses. Detection of apoptosis in cytospin preparation was performed by TUNEL assay as previously reported (Biroccio et al., 2002). In brief, 50 μ l of TUNEL reaction mixture was applied to the cytospin preparation, and the slides were incubated for 45 min at 37°C. Incorporated Bio-16-dUTP was then stained with

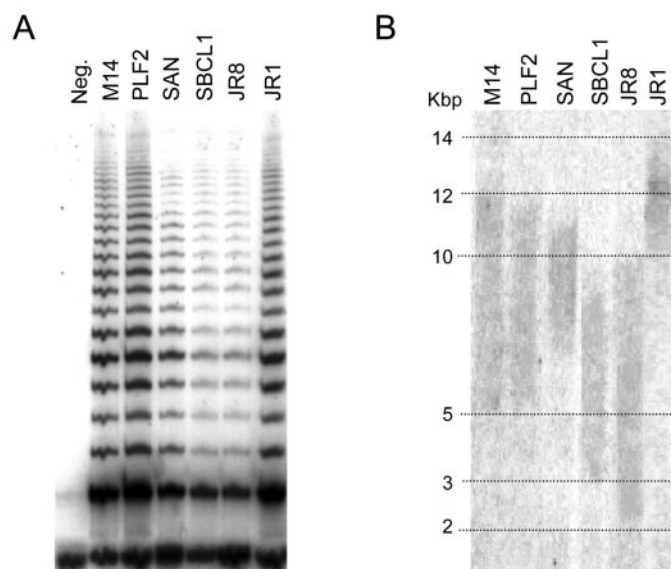


Fig. 2. Telomerase activity and telomere length in melanoma lines. A, TRAP assay to evaluate telomerase activity in M14, PLF2, JR1, JR8, SBCL1, and SAN human melanoma cells by using 0.5 μ g of total proteins. A sample without cell lysate (negative control, Neg) was included in the experiment. Similar results have been obtained by using 0.2 and 0.1 μ g of total protein (data not shown). B, terminal restriction fragment measured by Southern blot in M14, PLF2, JR1, JR8, SBCL1, and SAN human melanoma cells hybridized with the telomeric repeat (TTAGGG)₃. Representatives of three independent experiments are shown.

streptavidin-biotinylated horseradish peroxidase complex and visualized by using diaminobenzidine as chromogen. The percentage of apoptotic cells was determined by microscopic examination of TUNEL-treated slides at 40×. For each slide, five fields were examined, and 100 cells in each field were counted.

SA-β-gal staining was performed as reported previously (Biroccio et al., 2003a). In brief, cells were fixed with 2% glutaraldehyde in PBS for 5 min at room temperature, washed in PBS, and incubated for several hours in staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂ in PBS, pH 6.0.

Nuclei were stained with 1 μg/ml Hoechst 22358 and analyzed using a fluorescence microscope. The percentage of polynucleate cells and the presence of anaphase/telophase bridge were determined by microscopic examination of Hoechst-stained slides at 40×. At least five fields were examined for each slide.

Western Blotting. Western blot was performed as reported previously (Biroccio et al., 2002). Forty micrograms of total proteins were loaded from each sample on denaturing SDS-polyacrylamide gel electrophoresis. Detection of c-Myc was done using anti-c-myc monoclonal antibody (1:1000; clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence was used for detection. To check the amount of proteins transferred to nitrocellulose membrane, β-actin was used as control and detected by an anti-

human β-actin monoclonal antibody (1:500; Santa Cruz). The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad, Milano, Italy) and normalized to the related β-actin amounts.

Cytogenetic Analysis. Chromosome aberrations were evaluated as previously reported (Biroccio et al., 2003b). To obtain chromosome preparations, cells in the log phase of growth were incubated with 0.1 μg/ml colchicine for 2 h and trypsinized, then incubated with hypotonic 0.075 M KCl for 10 min, fixed with methanol/acetic acid (3:1, v/v), dropped onto frosted microscope slides, and air-dried overnight. Chromosomal aberrations were blindly evaluated by two independent observers in Giemsa-stained metaphases from two grown cultures for each line and each treatment. Analysis was performed at day 3 of treatment. For all the experiments, metaphase preparations of the different cells were performed simultaneously under the same conditions. The χ² test was used for statistical analysis.

TRAP Assay. Telomerase enzyme activity was measured with the polymerase chain reaction-based TRAP kit (Intergen Company, Oxford, UK), as reported previously (Biroccio et al., 2002). To define the sensitivity of the method and the semiquantitative relationship between protein concentration and ladder band intensity, different amounts of protein extract (from 0.01 to 2 μg) were used for each cell line and for all assays. In all cases reaction products were amplified in the presence of a 36-bp internal TRAP assay standard, and each

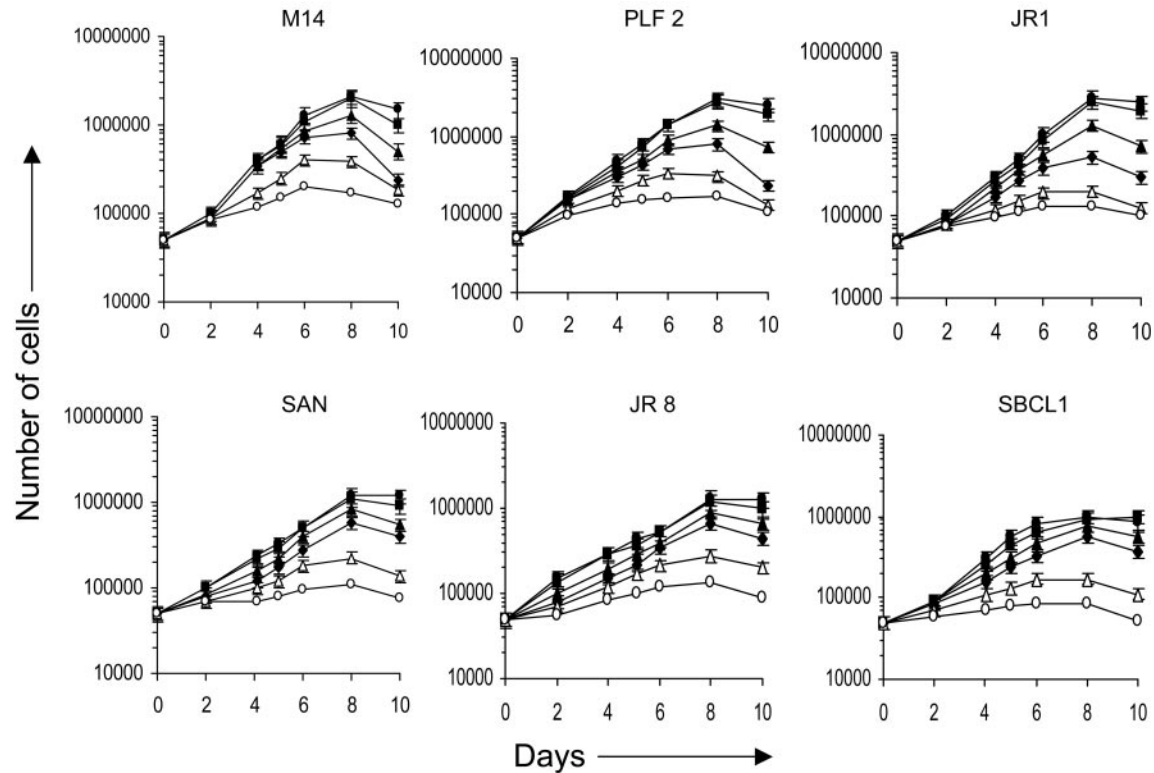


Fig. 3. Effect of RHPS4 on short-term cell proliferation. In vitro growth curves of the M14, PLF2, JR1, JR8, SBCL1, and SAN human melanoma cells untreated (●) and treated with 0.2 (■), 0.5 (▲), 1 (◆), 5 (△), and 10 (○) μM RHPS4. RHPS4 was added to the culture medium at days 1 and 5 of culture. The figure shows representative experiments performed in quintuplicate with standard deviations (SD).

TABLE 1
RHPS4 IC₅₀

The RHPS4 dose inhibiting cell proliferation by 50% (IC₅₀) was calculated at days 5 and 7 of treatment.

Day	Lines					
	M14	PLF2	JR1	SAN	JR8	SBCL1
	μM					
5	3.1 ± 0.5	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
7	1.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	1.1 ± 0.2	0.9 ± 0.2

extract was tested for heat sensitivity. Each set of TRAP assays included a control reaction without extract.

Southern Blotting. TRF determination was performed as reported previously (Biroccio et al., 2002). In brief, 15 μ g of DNA was digested with 40 units of HinfI and electrophoresed on 0.8% agarose gel. Then, DNA was denatured, neutralized, transferred to a nylon membrane (Hybond N; Amersham Biosciences, Buckinghamshire, UK) and cross-linked with ultraviolet light. The membrane was hybridized with 5'-end [γ - 32 P]deoxyadenosine triphosphate-labeled telomeric oligonucleotide probe (TTAGGG)₃ at 42°C for 2 h in a rapid hybridization buffer (QuikHyb hybridization solution; Stratagene, La Jolla, CA). After washing, the filters were autoradiographed (Hyperfilm MP; Amersham) with an intensifying screen at -80°C for 24 h; the autoradiographs were scanned, and the mean telomere length was calculated.

Results

RHPS4 Treatment Is Effective on Different Melanoma Lines with Long Telomeres. To evaluate the biological effects of RHPS4, a panel of six human melanoma lines (M14,

PLF2, JR1, JR8, SBCL1, and SAN), established in our laboratory, was used. SBCL1 cells apart, all the lines show a wild-type Rb pathway; moreover, M14, JR1, and JR8 cells possess a nonfunctional p53 protein, whereas an active p53 is present in PLF2, SAN, and SBCL1 lines (data not shown).

The different cell lines were first characterized in terms of telomerase activity and telomere length. TRAP assay, reported in Fig. 2A, demonstrated that all melanoma cell lines are telomerase-positive, even though telomerase activity is expressed at different levels. TRFs, measured by Southern blot (Fig. 2B), revealed that the lines possess relatively long telomeres; in fact, in JR8 cells, the line with the shortest telomeres, telomere length ranges from ~10 to ~2 kb, with a mean length of ~5 kb.

Figure 3 shows the short-term cell viability of M14, PLF2, JR1, JR8, SBCL1, and SAN cell lines treated with increasing RHPS4 concentrations, ranging from 0.2 to 10 μ M. No effect on the cellular growth is observed after treatment with RHPS4 at the dose of 0.2 μ M in all the lines employed. On the contrary, a

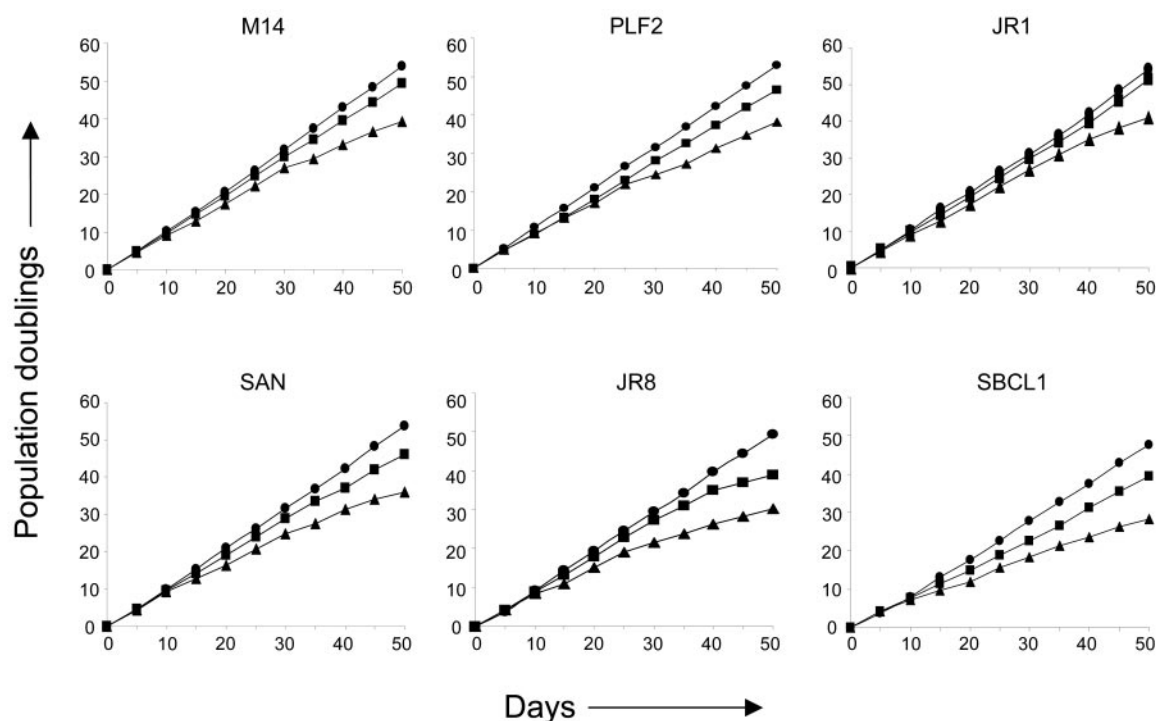


Fig. 4. Effect of RHPS4 on long-term cell proliferation. Proliferation (population doublings), measured as a function of time, of M14, PLF2, JR1, JR8, SBCL1, and SAN human melanoma cells untreated (●) and treated with 0.2 (■) and 0.5 (▲) μ M RHPS4. The data represent the mean of two independent experiments with standard deviation less than 10%.

TABLE 2

Telomeric fusions evaluated in M14 and JR8 cells at day 3 of RHPS4 treatment

Lines	RHPS4	No. of Cells Analyzed	No. of Cells with Telomeric Fusions						Total No. of Telomeric Fusions
			0	1	2	3	4	5	
M14	μ M								
	0	50	46	4					4
	0.5	40	8	14	10	7	1		59
	1	72	14	19	24	11	1	3	119
	5	51	3	13	11	14	9	1	118
JR8	10	46	3	11	12	10	8	2	107
	0	50	39	11					11
	0.5	46	13	22	10	1			45
	1	50	6	10	16	10	4	4	108
	5	50	5	11	18	8	5	3	106
	10	31	3	4	6	10	7	1	79

dose-dependent decrease of cell proliferation is evident after exposure to 0.5 and 1 μM RHPS4 during the days of growth. Furthermore, 5 and 10 μM concentrations cause a strong inhibition of cell proliferation at very early time of treatment. In Table 1 are reported the IC_{50} values for RHPS4, calculated by the proliferation assay. Across the different lines, in 5-day growth inhibition assays, the IC_{50} value is $\sim 3 \mu\text{M}$ for M14 cells, whereas it is $\sim 1 \mu\text{M}$ for the other lines. In a 7-day growth inhibition assay, the IC_{50} values are $\sim 1 \mu\text{M}$ for all the lines.

The lowest drug concentrations have been also used to evaluate the effect of RHPS4 on long-term cell viability (Fig. 4). Addition of 0.2 and 0.5 μM RHPS4 has no immediate antiproliferative effect on the melanoma growth. Growth rates, however, slow in a dose-dependent manner in all the lines employed; after 50 days of treatment, melanoma proliferation decreases by $\sim 35\%$ compared with untreated cells. TRF analysis, performed at the same day of treatment, demonstrates no detectable shortening of telomeres (data not shown).

RHPS4 Treatment Causes Telomere Dysfunction. The effect of RHPS4 on telomere integrity has been evaluated at very early time of treatment (day 3), when, at least for RHPS4 doses up to 1 μM , no effect on cell proliferation was observed. Telomere status was analyzed on viable M14 and JR8 cells untreated and treated with 0.5, 1, 5 and 10 μM RHPS4. No exchange figures or breaks are found both in M14 and JR8 cells after RHPS4 treatment. It is interesting that, as is evident from the row data reported in Table 2, RHPS4-treated cells have a high number of telomeric fusions. Telomeric fusion frequency (Fig. 5), calculated as total number of telomeric fusions/total number of metaphases, significantly ($p < 0.001$) enhances both in M14 and JR8 cells with the increasing drug concentration. In particular, the frequency of end-to-end fusion rises from ~ 1 to ~ 2.5 at 0.5 and 10 μM RHPS4, respectively.

Staining of nuclei with Hoechst dye, performed at day 7 of treatment, revealed that cells treated with RHPS4 contain multiple or multilobulated nuclei. The results reported in Fig. 6A demonstrate that the number of polynucleated cells rises with increasing drug concentrations, the percentage of cells ranging from 5 to 40% at 0.5 and 10 μM doses of RHPS4. Moreover, typical images of telophase bridge are found in RHPS4-treated cells. In Fig. 6B, nuclei of M14 cells treated or not with two different doses of RHPS4 are shown; polynucleated cells and typical images of telophase bridge are present.

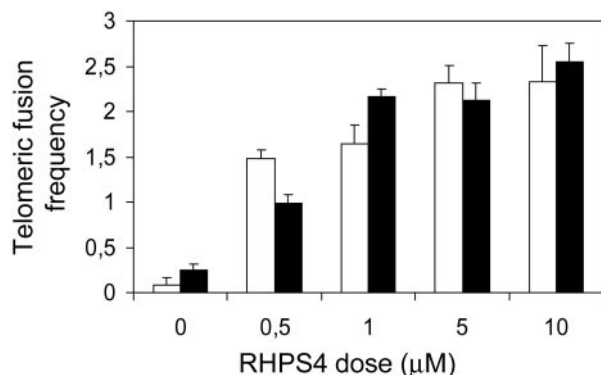


Fig. 5. Effect of RHPS4 on telomere status. Telomeric fusion frequency (calculated as total number of telomeric fusions/total number of metaphases) analyzed in M14 (\square) and JR8 (\blacksquare) cells untreated (0 μM) and treated with 0.5, 1, 5, and 10 μM RHPS4 for 3 days. The data represent the mean of three independent experiments with standard deviation (SD).

Telomerase activity and telomere length have been also evaluated in RHPS4-treated cells. Figure 7A shows a semi-quantitative TRAP assay performed in M14 cells treated or not with increasing doses of RHPS4. It is evident that, in agreement with results observed for porphyrin-based G-quadruplex inhibitors (Izbicka et al., 1999), RHPS4 treatment induces a reduction in cellular telomerase activity by $\sim 50\%$ only at the doses 5 and 10 μM . This effect is associated with the decrease of c-Myc levels. Western blot analysis (Fig. 7B), revealed that although no change in c-Myc protein is observed up to 1 μM , a dose-dependent decrease of c-Myc expression level was observed at the doses of 5 and 10 μM . The effect on telomerase activity is not accompanied by any reduction in telomere length. Southern blot analysis performed in M14 cells (Fig. 7C) demonstrated that TRF length in M14 cells treated or not with RHPS4 ranges from ~ 12 to ~ 5 kb, with a mean length of ~ 6 kb. Similar results have been obtained in the other lines (data not shown).

RHPS4 Treatment Induces Cell Cycle Perturbation, Apoptosis, and Senescence. To study whether RHPS4-induced telomere dysfunction results in alteration of cell cycle distribution, the percentage of cells in the different phases of cell cycle was analyzed by flow cytometry. Figure 8 shows the histograms of DNA content in M14 cells both untreated and treated with RHPS4 at doses of 0.5, 1, 5, and 10 μM . Analysis of cell percentages in the different phases of the cell cycle, performed from days 4 to 8 of treatment, revealed that RHPS4 induces a dose-dependent accumulation of cells in the S-G₂/M phase of the cell cycle, with a concomitant decrease in the G₀/G₁ compartment. This accumulation is very evident at day 6; the percentage of cells in the S-G₂/M phases of cell cycle was $\sim 34\%$ in control cells and ranged from 44 to 63% in cells treated with increasing dose of RHPS4 from 0.5 to 10 μM . Daily analysis demonstrated that control cells progressively accumulate in the G₀/G₁ phase of cell cycle, whereas the cells treated with RHPS4 still remain in the S-G₂/M compartment. These results suggest that RHPS4 induces a delay in progression through the cell cycle. Moreover, at day 8, the cells treated with RHPS4 show a strong perturbation of the cell cycle and a fraction of cell population resides to the subG₁ compartment.

On the basis of these results, apoptosis and senescence have been evaluated (Fig. 9). TUNEL assay, performed in M14 cells untreated and treated with RHPS4 at the doses of 0.5, 1, 5, and 10 μM , demonstrated that apoptosis is observed in RHPS4-treated cells at all doses of drug used. Moreover, consistent with the flow cytometric analysis performed at day 8, a higher number of TUNEL-positive cells are observed at 0.5 and 1 μM doses of RHPS4 compared with 5 and 10 μM . At 5 and 10 μM doses of RHPS4, apoptosis is accompanied by the induction of a senescence phenotype: large cell size, vacuolated cytoplasm, and β -galactosidase activity. Indeed, although only a few β -gal-positive cells are found in RHPS4-treated cells at doses of 0.5 and 1 μM , a high number of senescent cells are observed at doses of 5 and 10 μM . Figure 10 shows the percentage of TUNEL- and SA- β -gal-positive cells in M14, PLF2, and JR1 cells treated or not with RHPS4 at doses of 0.5, 1, 5, and 10 μM . It is evident that at 0.5 and 1 μM RHPS4 doses, ~ 35 and $\sim 40\%$ of cells are TUNEL-positive, respectively, a percentage that decreases at doses of 5 and 10 μM . Moreover, a progressive increase in the percentage of senescent cells is found with the rising of drug concentrations, the percentage of SA- β -gal-positive cells reach-

ing the significant values of ~ 40 and $\sim 60\%$ at 5 and 10 μM doses of RHPS4, respectively.

Discussion

In this study, we have used a second-generation small molecule capable of interacting with G-quadruplexes, the pentacyclic acridine RHPS4, which possesses a high selectivity for quadruplex DNA structure (Gowan et al., 2001; Heald et al., 2002).

We demonstrate that short-term RHPS4 treatment of telomerase-positive melanoma lines, possessing long telomeres,

causes a marked inhibition of cell proliferation in a dose-dependent manner. This effect is caused mainly by telomere dysfunction. In fact, at very early times of treatment, when cell proliferation was unaffected, a significant increase of telomeric fusions was found in RHPS4-treated cells. Moreover, polynucleated cells and typical images of telophase bridge, as markers of telomere dysfunction, were also observed in cells treated with the drug. On the other hand, no effect on telomere length has been found in cells exposed to different RHPS4 doses for either short or long exposures (up to 50 days). However, we cannot exclude the possibility that subtle changes in telomere length

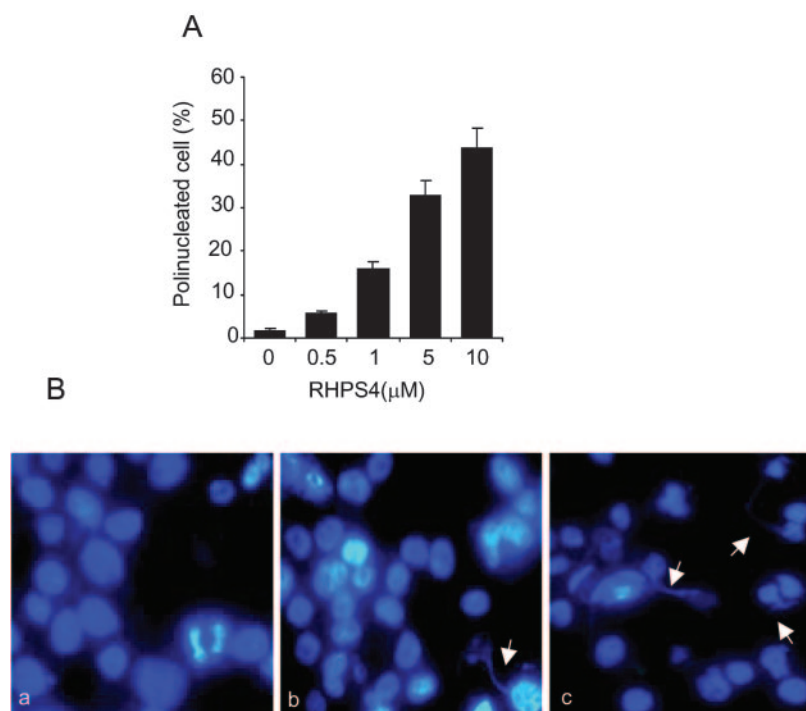


Fig. 6. Effect of RHPS4 on nuclear stability. A, percentage of polynucleated cells analyzed by Hoechst staining in M14 cells untreated (0 μM) and treated with 0.5, 1, 5, and 10 μM RHPS4. The analysis was performed at day 7 of treatment. The data represent the mean of four independent experiments with standard deviations (SD). B, Hoechst staining of M14 cells untreated (a) and treated with 1 (b) and 10 μM (c) of RHPS4. The analysis was performed at day 7 of treatment. Arrows indicate telophase bridges. A representative of three independent experiments with comparable results is shown. Original magnification, 200 \times .

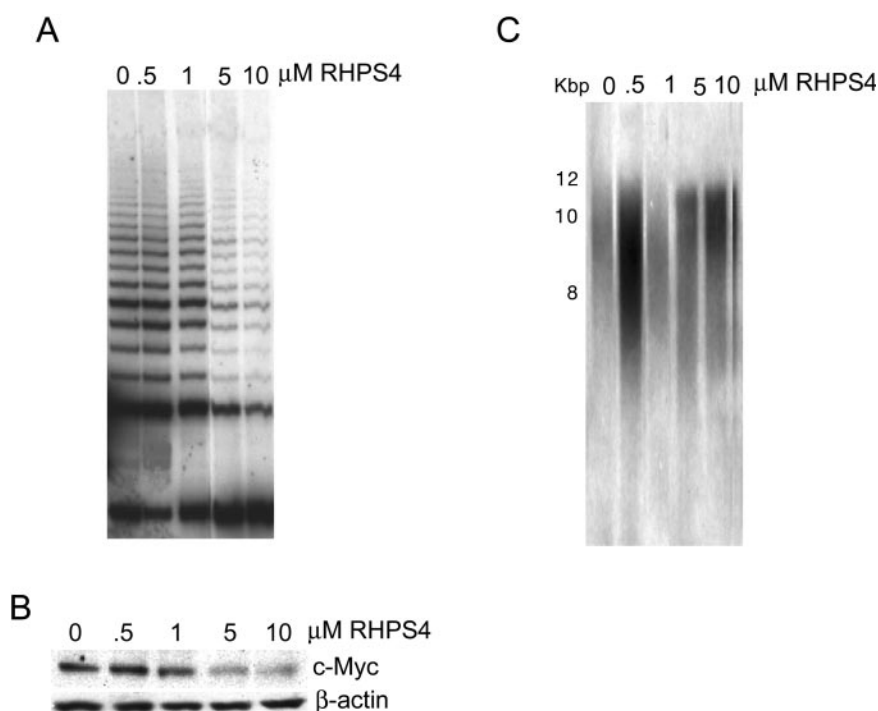


Fig. 7. Effect of RHPS4 on telomerase activity, c-Myc expression, and telomere length. A, TRAP assay to evaluate telomerase activity in M14 cells untreated (0 μM) and treated with 0.5, 1, 5, and 10 μM RHPS4. The analysis was performed using 0.5 μg of total proteins, and a sample without cell lysate (negative control, Neg) was included in the experiment. Same results have been obtained by using 0.2 and 0.1 μg of total proteins (data not shown). B, Western blot analysis of c-Myc protein expression performed in M14 cells untreated (0 μM) and treated with 0.5, 1, 5, and 10 μM RHPS4. β -Actin was used as control for protein loading. C, TRF measured by Southern blot in M14 cells untreated (0 μM) and treated with 0.5, 1, 5, and 10 μM RHPS4. All the analyses were performed at day 7 of treatment, and representatives of three independent experiments are shown.

occurred, but they were not detectable by Southern blot. In fact, considering the initial mean and broad range of telomere length of all the melanoma lines, appreciable telomere shortening may require a much longer time of treatment. On the other hand, the reduction of cell viability observed in long-term experiments could be attributable to the ability of RHPS4 to preferentially induce telomere dysfunction. The change in growth rate observed in long-term experiments with the 0.5 μ M RHPS4 dose seems to be in contrast to the rapid effect seen in short-term assays. This can be explained by the survival advantage of cells with RHPS4-undamaged telomeres during culture passages.

Our data concur with recent findings demonstrating that

changes in telomere uncapping versus capping status may be as important as actual telomere length in determining cell survival or death (Blackburn, 2000; Kim et al., 2001). Moreover, similar findings were recently published for other G-quadruplex ligands, such as triazine (Riou et al., 2002) and porphyrin derivatives (Kim et al., 2003); however, contrasting findings were noted for telomestatin (Kim et al., 2003). The latter 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 (Kim et al., 2003). Based on these recent findings, it is possible that RHPS4 induces telomere

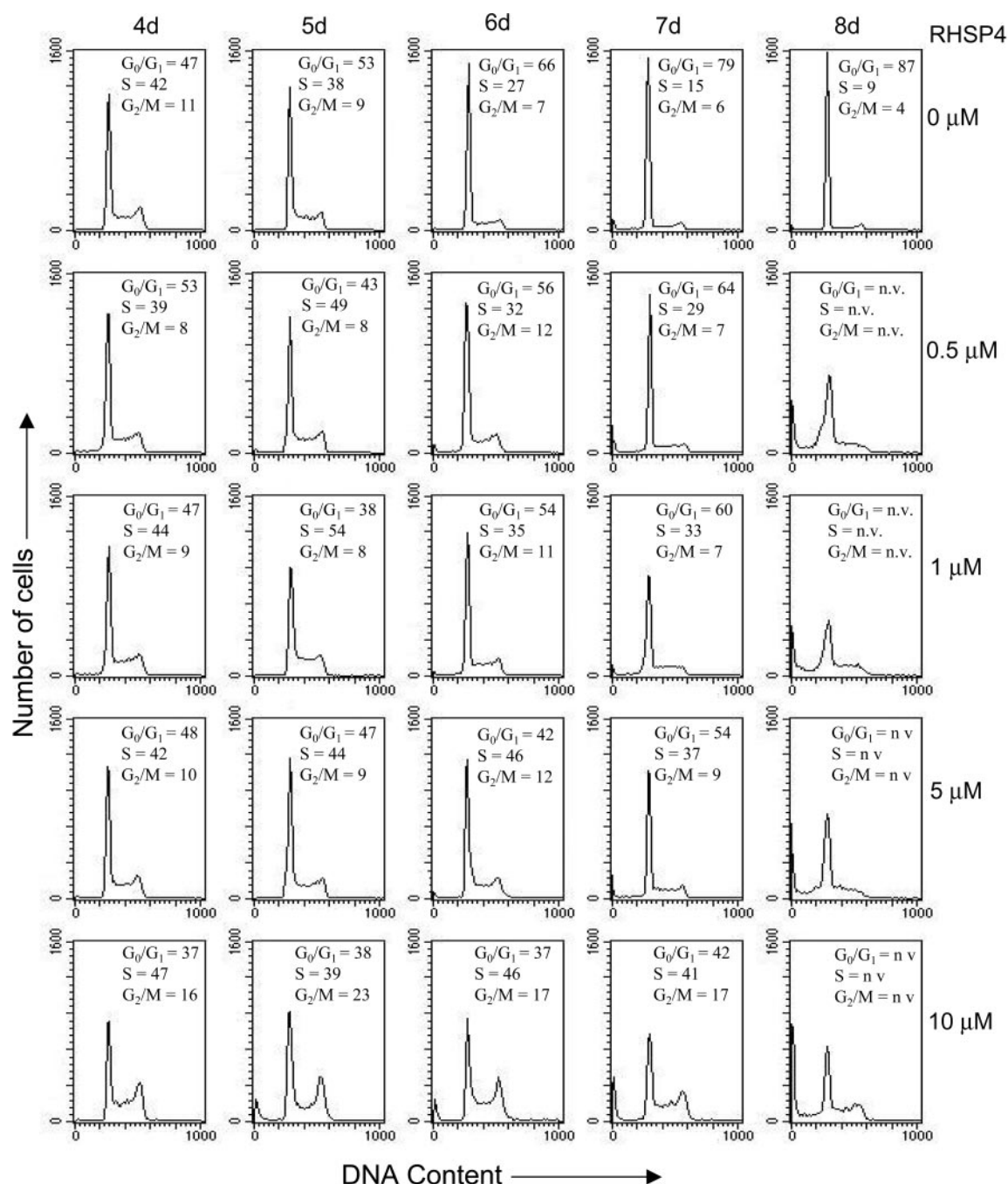


Fig. 8. Effect of RHPS4 on cell cycle of M14 cells. Cell cycle analysis after propidium iodide staining was performed by flow cytometry at days 4 to 8 of treatment with 0.5, 1, 5, and 10 μ M RHPS4. The percentages of cells in the different phases of cell cycle were reported inside the relative histogram. A representative of three independent experiments is shown.

uncapping through its ability to selectively interact with intermolecular rather than intramolecular G-quadruplex structures. The RHPS4-induced telomere uncapping is consistent with other results (Gowan et al., 2001), which demonstrated that biological effects of RHPS4 on A421 and 21NT tumor cells possessing short telomeres, are not associated with any detectable decrease in telomere length. Taken together, the present and earlier results (Gowan et al., 2001), suggest that the G-quadruplex ligand RHPS4 can work in a telomere length-independent manner through its ability to induce telomere-capping alteration.

We also report here that the short-term cellular effects of RHPS4 are associated with a dose-dependent accumulation of cells in the S-G₂/M compartment of the cell cycle. The RHPS4-induced cell cycle alteration is irreversible even at lowest doses (0.5 and 1 μ M), and the cells die from apoptosis. RHPS4-induced apoptosis does not seem to be dependent on functional p53 pathway. In fact, whereas PLF2, SAN, and SBCL1 have an active p53 protein, M14, JR1, and JR8 cells possess a nonfunctional p53, suggesting that an intact p53 pathway is not required for RHPS4-induced apoptosis. At high RHPS4 doses (5 and 10 μ M), apoptosis was accompanied by the induction of a senescence phenotype: large cell size, vacuolated cytoplasm, and β -galactosidase activity. The RHPS4-induced senescence is associated with a reduction in telomerase activity in cells incubated with the G-quadruplex ligand. Indeed, although no decrease in cellular telomerase activity has been observed at concentrations of RHPS4 up to 1 μ M, a significant inhibition of telomerase is observed at

higher doses. RHPS4-mediated reduction in telomerase activity is not a surprising finding, because a similar effect has been also observed with porphyrin-based G-quadruplex interactive telomerase inhibitors (Izbicka et al., 1999). In agreement with the results obtained using the cationic porphyrin TMPyP4, the ability of RHPS4 to down-regulate telomerase activity may occur via effects on *c-myc*. Indeed, a reduction of c-Myc protein expression, concomitantly with a decrease of telomerase activity, has been observed. The effects on telomerase activity and c-Myc could be caused by 1) an direct interaction of RHPS4 with the quadruplex-sensitive promoter region of the *c-myc* gene (Rangan et al., 2001), which has been shown to regulate hTERT (Wang et al., 1998); 2) a nonspecific duplex binding; and 3) a consequence of the RHPS4-mediated inhibition of cell proliferation. More-

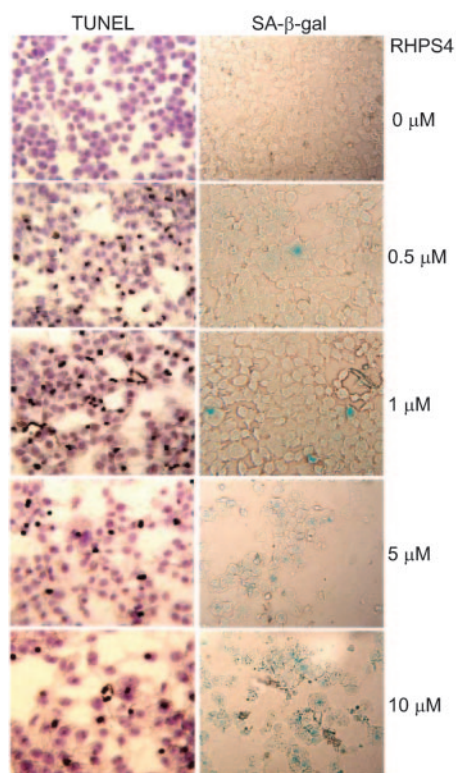


Fig. 9. Effect of RHPS4 on the induction of apoptosis and senescence phenotype in M14 cells. TUNEL and SA- β -gal staining of M14 cells untreated (0 μ M) and treated with 0.5, 1, 5, and 10 μ M RHPS4. The analysis was performed at day 7 of treatment. A representative of three independent experiments with comparable results is shown. Original magnification, 40 \times .

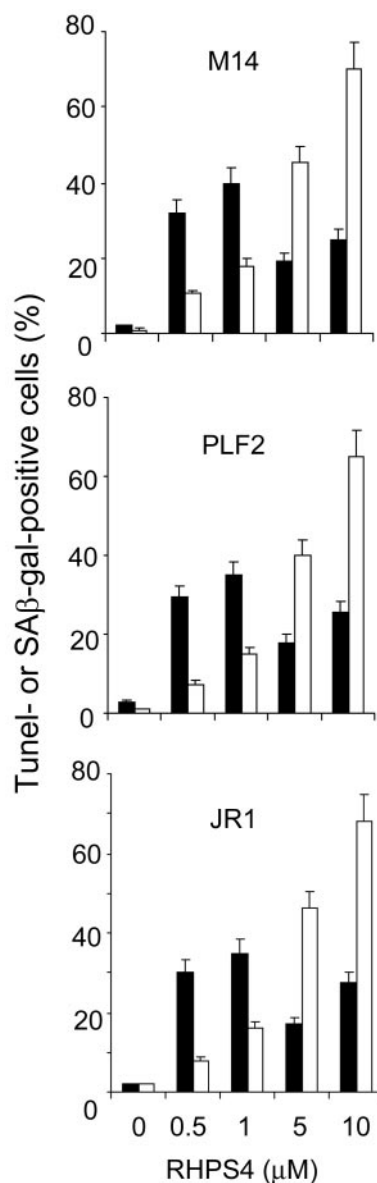


Fig. 10. Effect of RHPS4 on apoptosis and senescence in melanoma lines. Percentage of TUNEL-positive (■) and SA- β -gal-positive cells (□) in M14, PLF2, and JR1 cell lines untreated (0 μ M) and treated with 0.5, 1, 5, and 10 μ M RHPS4. The analysis was performed at day 7 of treatment. The data represent the mean of four independent experiments with standard deviations (SD).

over, because the inhibition of cellular telomerase activity was observed only at RHPS4 concentrations of 5 and 10 μM , we can conclude that RHPS4-induced apoptosis, observed at 0.5 and 1 μM , is independent of the ability of RHPS4 to induce inhibition of the in vitro telomerase activity. On the other hand, the telomerase inhibition observed at high doses could contribute to the increased susceptibility of cells to RHPS4. Our results are in agreement with recent reports showing that telomerase overexpression can suppress DNA-damage or damage-related signals that trigger cell death or senescence (Oh et al., 2001; Akiyama et al., 2002). Moreover, because it has been demonstrated that telomerase itself, in addition to elongating telomeric DNA, can protect telomeres from being damaged (Blackburn, 2000), telomerase could be essential in RHPS4-treated cells with alteration of telomere-capping functions. This explanation is consistent with recent findings showing that transfection of dominant-negative hTERT cDNA into cells resistant to the G-quadruplex ligand 12459, showing telomerase overexpression and telomere-capping alteration, restores the sensitivity to apoptotic concentrations of ligand 12459 (Gomez et al., 2003).

In conclusion, our results demonstrate that the G-quadruplex ligand RHPS4 is effective on human melanoma lines possessing relatively long telomeres and that its biological effects, apoptosis and/or senescence, are associated with telomere-capping alteration. These interesting findings, supported by recent papers demonstrating the in vivo efficacy of the tri-substitute acridine BRACO-19 in combination with paclitaxel (Gowan et al., 2002), suggest that the short-term use of RHPS4 in combination with cytotoxic drugs may represent a good clinical strategy to achieve effective killing of the tumor cell population.

Acknowledgments

We thank Adele Petricca for her helpful assistance in typing the manuscript.

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