Inhibition of telomerase activity by geldanamycin and 17-allylamino, 17-demethoxygeldanamycin in human melanoma cells

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As it has been demonstrated that the heat shock protein 90 (HSP90) is required for the assembly and activation of telomerase in human cells, we investigated the effect exerted by the ansamycin antibiotics geldanamycin (GA) and 17-allylamino,17-demethoxygeldanamycin (17-AAG), two well-known inhibitors of the HSP90 chaperone function, on telomerase activity in JR8 human melanoma cells. Using an antibody to HSP90, we precipitated the telomerase activity associated with the molecular chaperone. The results of TRAP (telomeric repeat amplification protocol) experiments carried out on HSP90 immunoprecipitates showed that exposure to 100 ng/ml GA and 17-AAG induced a significant (P < 0.01) inhibition of telomerase activity, which was observed at earlier time points than drug-induced inhibition of cell proliferation. Superimposable results were obtained from TRAP experiments carried out on total JR8 protein extracts. To investigate whether the basal level of telomerase activity of the tumour cell system plays a role in determining the cellular response to 17-AAG, we compared the cytotoxic activity of the drug in JR8 cells and in two JR8-derived clones that were stably transfected with a hammerhead ribozyme targeting the RNA template of telomerase and were characterized by a markedly lower telomerase activity than the parental cells. The cytotoxicity results indicated that both ribozyme-transfected clones were almost 2-fold more sensitive to 72 h 17-AAG exposure than JR8 cells as a consequence of a more than double apoptotic response [in terms of the percentage of apoptotic nuclei in cells stained with propidium iodide and the percentage of Tdt-mediated dUTP nick-end labelling (TUNEL)-positive cells]. In summary, our results suggest that (i) telomerase is a target of GA and 17-AAG action and its inhibition may contribute to the cytotoxic activity of the drugs, (ii) the basal level of telomerase activity of the tumour cell system may also have a role in influencing 17-AAG cytotoxicity.

Introduction

The benzoquinone ansamycin antibiotic geldanamycin (GA) and its derivative 17-allylamino,17-demethoxygeldanamycin (17-AAG) are inhibitors of the heat shock protein 90 (HSP90) (1,2), a molecular chaperone that plays a role in protein refolding in cells exposed to environmental stress (3,4) and is required for conformational maturation, stability and activity of several proteins involved in signal transduction pathways (5). Specifically, HSP90 is less promiscuous than other chaperones and its client proteins include receptor and non-receptor kinases (HER-2, epidermal growth factor receptor and Src family kinases), serine/threonine kinases (c-Raf-1 and Cdk4), steroid hormone receptors (androgen and estrogen), and cell cycle and apoptosis regulators (mutated p53) (6–12), all of which play a role in promoting the growth and/or survival of cancer cells. By forming conformation-dependent high-order chaperone complexes, HSP90 regulates the half-lives of its client proteins (13). The interaction of GA and 17-AAG with HSP90 results in competition for ATP binding to HSP90 and inhibition of its chaperone functions, leading to destabilization and proteosomal degradation of the client proteins (14). These compounds have shown promising antitumour activity in several pre-clinical models (15–19), and 17-AAG, which is characterized by reduced liver toxicity with respect to its parent compound (20), is currently undergoing Phase I clinical trials. Recent studies have shown that HSP90 and the co-chaperone p23 are required for efficient telomerase assembly in a cell-free system and in intact human cells (21). The proposed model suggests that both HSP90 and p23 bind to the telomerase reverse transcriptase hTERT and influence proper assembly with the telomerase RNA template hTR to form an active enzyme (22). Telomerase is a ribonucleoprotein that stabilizes telomere length in tumour cells, thus preventing replicative senescence (23). Moreover, it seems to play a crucial role in capping and protecting the telomere from signalling into cell-cycle arrest and/or apoptosis (24). The notion that telomerase is expressed in ~85–90% of human tumours makes it a promising therapeutic target for novel anticancer treatments and distinct rationales for the development of specific inhibitors have been formulated on the basis of the understanding of the composition and functions of the enzyme. Such inhibitors include antisense molecules and ribozymes targeting the telomerase subunits hTR and hTERT (25), reverse transcriptase inhibitors (26), hTERT dominant negative mutants (27) and small molecules interacting with G-quadruplexes in telomeric DNA (28).

In this study we demonstrated that it is possible to down-regulate telomerase activity through the interference with HSP90 function in human tumour cells. Specifically, exposure of JR8 melanoma cells to GA and 17-AAG induced marked inhibition of the enzyme’s catalytic activity, which was appreciable at earlier time-points than the drug-induced antiproliferative effect. Moreover, two JR8 clones, stably transfected with a hammerhead ribozyme targeting hTR and characterized by a significantly lower basal telomerase activity than the parental cells (29), showed an almost 2-fold increased sensitivity to the cytotoxic effect of 17-AAG with respect to JR8.

Abbreviations: 17-AAG, 17-allylamino,17-demethoxygeldanamycin; GA, geldanamycin; HSP90, heat shock protein 90; SRB, Sulforhodamine B; TRAP, telomeric repeat amplification protocol; TUNEL, Tdt-mediated dUTP nick-end labelling.

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cells, suggesting that telomerase might play a role in influencing the cellular response to 17-AAG.

Materials and methods

Cell line and ribozyme-transfected clones

The JR8 human melanoma cell line was maintained in the logarithmic growth phase at 37°C in a 5% CO₂ humidified atmosphere in air, using the RPMI-1640 medium (Bio-Wittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel), 2 mM t-glutamine and 0.12% gentamicin. As described previously (29), the JR8 cell line was transfected with the pRc/CMV expression vector containing the sequence of a hammerhead ribozyme targeting the RNA template of human telomerase (30). Ribozyme-transfected clones were selected in vitro by G418. Two clones, JR8pRcRzB2 and JR8pRcRzB15, proven to endogenously express the ribozyme and characterized by a markedly lower telomerase activity than the parental cells (29), and one clone, JR8rcCMV, transfected with the empty vector, were used in this study.

Drugs

Geldanamycin (GA) (Sigma Chemical Co., St Louis, MO) and 17-allylamino,17-deoxymethoxygeldanamycin (17-AAG) (kindly provided by Dr E. Saussion, NICI, Bethesda, MA) were reconstituted in sterile DMSO at a concentration of 5 mM and then diluted with sterile water to the desired concentrations immediately before each experiment.

Cell survival assay

The Sulforhodamine B (SRB) assay was performed as described by Perez et al. (31) with minor modifications. Briefly, according to the growth profiles preliminarily defined for each cellular model, adequate numbers of cells in 0.2 ml culture medium were plated in each well of a 96-well plate and allowed to attach for 24 h. JR8 cells were exposed to 10, 20, 60 or 100 ng/ml GA or 17-AAG at 37°C for 24, 48 and 72 h, whereas ribozyme-transfected clones were exposed to the same drug concentrations for 72 h. In each experiment, control samples were run with 0.3% DMSO. At the end of each treatment, cells were exposed to the same drug concentrations for 72 h. In each experiment, control samples were run with 0.3% DMSO. At the end of each treatment, cells were harvested and fixed in 0.1% citrate for 2 min in ice. Samples washed with PBS were then incubated in Tdt-mediated dUTP nick-end labelling (TUNEL) analysis for 1 h at 37°C, which was followed by 4× 10 s pulse sonication at 50 J/Watt-s, alternated by 30 s intervals on ice. The lysates were then spun at 13 000 r.p.m. for 15 min at 4°C, and the resulting supernatants were used for immunoprecipitation. Sixteen micrograms of rabbit polyclonal HSP90 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were pre-coupled to 8 μl of a 50% slurry of protein-G-agarose beads by incubating for 1 h at 4°C, with constant rotation. The antibody-coated beads were washed extensively with lysin buffer prior to use in immunoprecipitation reactions. Four microliters of cell lysate and 16 μl of 5% BSA (in lysin buffer) were combined with antibody beads and rotated for 1 h at 4°C. Immunoprecipitates were then washed with the lysin buffer 4× 350 μl with rotation at 4°C. For the TRAP (telomeric repeat amplification protocol) assay following immunoprecipitation, protein G-agarose pellets were resuspended in a final volume of 8 μl with lysin buffer and 4 μl was removed for the TRAP assay.

Evaluation of apoptotic morphology by fluorescence microscopy

Cells exposed to different concentrations (10, 20 and 60 ng/ml) of 17-AAG for 72 h were harvested, washed in PBS and stained with solution A. After staining, the cells on the slides were examined by fluorescence microscopy. The percentage of apoptotic cells was determined by scoring at least 500 cells in each sample.

Immunoprecipitation

Immunoprecipitation was performed as described by Holt et al. (21). JR8 cells were suspended at a concentration of 1×10⁶ cells/ml in lysis buffer (0.01% NP-40, 10 mM Tris pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2 mM DTT, 20% glycerol plus protease inhibitors) and incubated on ice for 20 min, this was followed by 4× 10 s pulse sonication at 50 J/Watt-s, alternated by 30 s intervals on ice. The lysates were then spun at 13 000 r.p.m. for 15 min at 4°C, and the resulting supernatants were used for immunoprecipitation. Sixteen micrograms of rabbit polyclonal HSP90 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were pre-coupled to 8 μl of a 50% slurry of protein-G-agarose beads by incubating for 1 h at 4°C, with constant rotation. The antibody-coated beads were washed extensively with lysin buffer prior to use in immunoprecipitation reactions. Four microliters of cell lysate and 16 μl of 5% BSA (in lysin buffer) were combined with antibody beads and rotated for 1 h at 4°C. Immunoprecipitates were then washed with the lysin buffer 4× 350 μl with rotation at 4°C. For the TRAP (telomeric repeat amplification protocol) assay following immunoprecipitation, protein G-agarose pellets were resuspended in a final volume of 8 μl with lysin buffer and 4 μl was removed for the TRAP assay.

Tdt-mediated dUTP nick-end labelling (TUNEL) analysis

After 72 h exposure to 17-AAG (60 ng/ml), cells were harvested and fixed in 4% paraformaldehyde for 45 min at room temperature. After rinsing with PBS, the cells were permeabilized in a solution of 0.1% Triton X-100 in sodium 0.1% citrate for 2 min in ice. Samples washed with PBS were then incubated in the TUNEL reaction mixture (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C in the dark, and after rinsing with PBS they were suspended in PBS and analysed by a FACScan flow fluorometer (Becton Dickinson). The results were expressed as the percentage of TUNEL-positive cells in the overall cell population.

Continuous exposure of JR8 cells to 10–100 ng/ml of GA and 17-AAG for 24–72 h resulted in a dose- and time-dependent inhibition of cell growth, as determined by the SRB assay. The extent of such inhibition was almost negligible after 24 h of drug treatment and progressively increased with time for both drugs (Figure 1). Moreover, a greater cytotoxic effect was observed for 17-AAG than GA, as indicated by the lower IC₅₀ value calculated from the growth inhibition curves obtained after 72 h of treatment (31.2 ± 4.9 versus 48.0 ± 4.6 ng/ml; P < 0.05, Student’s t-test).

Flow cytometric analysis of cell-cycle progression in cells treated with GA (20–100 ng/ml) showed a dose-dependent accumulation of cells in the G₀ phase at 24 h, which was followed by an increase in the S-phase cell fraction at 48 h (Figure 2). Similar cell-cycle perturbations were observed after treatment with 17-AAG at the two lowest concentrations (20 and 60 ng/ml). Conversely, in cells exposed to the highest 17-AAG concentration (100 ng/ml), cell accumulation in the G₀ phase was persistent and still appreciable at 72 h (Figure 2).
Inhibition of telomerase by GA and 17-AAG

Continuous exposure to drugs induced a significant \( P < 0.01 \), Student's \( t \)-test) and stable inhibition of the telomerase catalytic activity, which was around \(-70\%\) of control with GA (Figure 5A) and \(-90\%\) of control with 17-AAG (Figure 6A). Moreover, the extent of telomerase activity inhibition was dependent on the drug concentration. In fact, exposure of JR8 cells to different drug concentrations (from 20 to 100 ng/ml) for 48 h induced a progressive decrease in the enzyme’s catalytic activity ranging from \(-1\%\) to \(-62\%\) of control with GA, and from \(-17\%\) to \(-89\%\) of control with 17-AAG (Figure 7). The results of the TRAP experiments performed on total cell extracts also confirmed the reversibility of telomerase inhibition upon drug removal, since a progressive increase in the TRAP signal was observed when the incubation time in drug-free medium was prolonged, with the level of telomerase activity in treated cells approaching that of control samples at 48 h from the end of treatment (Figures 5B and 6B).

To evaluate the long-term effect of drug-induced telomerase inhibition, we chronically exposed JR8 cells to GA or 17-AAG (60 ng/ml of drug once every 3 days for 21 days). However, we failed to observe any telomere shortening in drug-treated cells compared with controls, as detected by Southern blot analysis of telomere restriction fragments (Figure 8).

As it was previously suggested that the basal level of telomerase activity of the cellular model may affect the sensitivity to 17-AAG (35), in a further step of the study we compared the cytotoxic activity of 17-AAG in JR8 cells and in two JR8-derived cell clones (JR8pRcRzB2 and JR8pRcRzB15), stably transfected with a hammerhead ribozyme targeting the RNA template of telomerase and characterized by a markedly lower telomerase activity than that of JR8 parental cells (\(-76\%\) for JR8pRcRzB15 and \(-95\%\) for JR8pRcRzB2) and the vector transfectant clone JR8pRcCMV (29). The results of SRB experiments showed that both clones were twice as sensitive to 72 h exposure to 17-AAG as JR8, as indicated by the reduced IC\(_{50}\) values (16.3 ± 1.5 ng/ml for JR8pRcRzB2 and 17.5 ± 3.1 ng/ml for JR8pRcRzB15 versus 31.2 ± 4.9 ng/ml for JR8; \( P < 0.05\), Student’s \( t \)-test). Conversely, the vector transfectant clone exhibited a sensitivity to 17-AAG comparable with that of JR8 parental cells (IC\(_{50}\): 40.0 ± 9.1 ng/ml).

As several lines of evidence suggest that telomerase is involved in the cellular resistance to apoptosis, we investigated whether or not the increased sensitivity to 17-AAG observed in ribozyme-expressing clones was due to an enhanced susceptibility to undergo apoptosis after drug treatment. When the presence of cells with an apoptotic nuclear morphology was determined by fluorescence microscopy after cell staining with propidium iodide (Figure 9A), a very limited percentage of apoptotic cells was observed in untreated JR8, JR8pRcRzB2 and JR8pRcRzB15 cells. These percentages progressively increased in treated samples as a function of 17-AAG concentration in all cell models. However, at the highest drug concentration (60 ng/ml) the extent of drug-induced apoptosis was significantly higher (\( P < 0.05\), Student’s \( t \)-test) in ribozyme-expressing clones than in JR8 parental cells (Figure 9B). In addition, the presence of drug-induced apoptosis in cells exposed to 60 ng/ml 17-AAG was determined by TUNEL analysis. DNA fragmentation in ribozyme-expressing clones was more marked than in parental cells, as indicated by the percentage of TUNEL-positive cells, which was higher in JR8pRcRzB2 (38.4%) and JR8pRcRzB15 (19.4%) compared with JR8 cells (7.7%) (Figure 10). Taken together, these results indicate that a reduced basal level of telomerase continuous exposure to drugs induced a significant \( P < 0.01 \), Student’s \( t \)-test) and stable inhibition of the telomerase catalytic activity, which was around \(-70\%\) of control with GA (Figure 5A) and \(-90\%\) of control with 17-AAG (Figure 6A). Moreover, the extent of telomerase activity inhibition was dependent on the drug concentration. In fact, exposure of JR8 cells to different drug concentrations (from 20 to 100 ng/ml) for 48 h induced a progressive decrease in the enzyme’s catalytic activity ranging from \(-1\%\) to \(-62\%\) of control with GA, and from \(-17\%\) to \(-89\%\) of control with 17-AAG (Figure 7). The results of the TRAP experiments performed on total cell extracts also confirmed the reversibility of telomerase inhibition upon drug removal, since a progressive increase in the TRAP signal was observed when the incubation time in drug-free medium was prolonged, with the level of telomerase activity in treated cells approaching that of control samples at 48 h from the end of treatment (Figures 5B and 6B).

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catalytic activity renders cells more prone to 17-AAG-induced apoptosis.

Discussion

In the present study we evaluated the effects induced by the ansamycin antibiotics GA and 17-AAG, two well-known inhibitors of the chaperone function of HSP90 (1,2), in JR8 human melanoma cells. Both drugs were able to inhibit cell growth as a function of concentration and exposure time. However, in accordance with previously published results (36), 17-AAG appeared more potent than the parent compound GA. Both drugs induced an accumulation of cells in the G1 phase, which was transient (only appreciable at 24 h) in cells

Fig. 2. Distribution of cells in different phases of the cell cycle (G1, S, G2M) after treatment with different concentrations of GA and 17-AAG for 24, 48 and 72 h in untreated samples (■) and samples exposed to 20 (●), 60 (▲) or 100 ng/ml (▼) of drugs.

Fig. 3. Effect of GA treatment on HSP90-associated telomerase activity in JR8 cells. (A) A representative TRAP experiment is shown. Untreated cells (lane 1); cells exposed to 100 ng/ml GA for 24 h and processed immediately (lane 2) or after 24 (lane 3) and 48 h (lane 4) in drug-free medium; cells continuously exposed to GA for 48 (lane 5) or 72 h (lane 6). (B) Quantification of telomerase activity of JR8 cells continuously exposed to 100 ng/ml GA for 24, 48 or 72 h. (C) Quantification of telomerase activity at different intervals in drug-free medium after 24 h exposure to GA. Data represent mean values (±SD) of three independent experiments.

Discussion

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exposed to the different GA concentrations, and became stable (still appreciable at 72 h) in cells exposed to the highest 17-AAG concentration (100 ng/ml). Although G1 phase cell accumulation was observed before inhibition of cell proliferation became apparent (24 versus 48 h), the extent of such an accumulation was dependent on drug concentration and related to the level of cell growth inhibition induced by drugs. The induction of G1 cell-cycle arrest by another ansamycin antibiotic, herbimycin A, was described by Srethapakdi *et al.* (37) in a series of retinoblastoma gene product (RB)-positive cell lines (like JR8), which was accompanied by hypophosphorylation of RB and downregulation of cyclin D- and E-associated kinase activities.

It has been demonstrated recently that HSP90 is required for telomerase activation. Specifically, Holt *et al.* (21) showed that the assembly of active telomerase from *in vitro*-synthesized core components (hTERT and hTR) requires the contribution of proteins present in rabbit reticulocyte extract. Such proteins, which have been identified as the molecular chaperones HSP90 and p23, bind to the catalytic subunit of telomerase.
hTERT. The blockade of such interaction by the HSP90 inhibitor GA inhibited the assembly of active telomerase in the rabbit reticulocyte system (21). Moreover, when quiescent HT1080 human fibrosarcoma cells showing downregulated telomerase by serum starvation were exposed to serum plus GA (100 ng/ml or greater), they failed to express active telomerase in response to serum, thus suggesting that HSP90 is required for induction of active telomerase also in vivo. The working model proposed by Forsythe et al. (22) suggests that the HSP90 chaperone complex serves to recruit hTR to hTERT working model proposed by Forsythe required for induction of active telomerase also in vivo of HSP90 and p23 with the functioning telomerase was observed in an in vitro telomerase assembly/reconstitution system, these authors also presumed that the additional ‘tweaking’ of conformation of the assembled complex required during the translocation step of telomerase action could be provided by the stably associated HSP90 and p23 (22). Interestingly, Akalin et al. (38) recently demonstrated that the expression of the HSP90 chaperone complex is enhanced during malignant transformation of prostate cells and in advanced prostate cancers compared with surrounding non-cancerous tissues, suggesting that up-regulation of this chaperone complex may have a role in the telomerase activation observed in cancer cells.

Based on these findings we investigated the possible effect exerted by GA and 17-AAG exposure on telomerase in JR8 melanoma cells. Using a polyclonal antibody to HSP90, we immunoprecipitated a significant fraction of telomerase activity from JR8 protein extracts and demonstrated that exposure to GA and 17-AAG induced marked inhibition of the enzyme activity associated with HSP90, which was slowly reversible upon drug removal. Moreover, the extent of telomerase inhibition was slightly higher for 17-AAG than for GA. Superimposable results were obtained when telomerase inhibition was measured on total extracts obtained from drug-treated JR8 cells. Although our data do not allow definition of the precise mechanism of telomerase inhibition by GA and 17-AAG, it appears that these drugs may inhibit the basic catalytic steps involved in template copying since the different TRAP products (as measured by densitometric analysis) were almost equally affected by the treatment. This finding could reflect a reduced abundance of catalytically active telomerase holoenzyme as a consequence of drug-induced impairment of HSP90 chaperone function. Telomerase inhibition does not seem to represent a secondary event of drug-induced cell arrest, as it was observed before the inhibition of cell proliferation became apparent (24 versus 48 h). Again, telomerase inhibition does not appear to be a consequence of drug-induced cell-cycle impairment, as it was also observed when the distribution of cells in the different cycle phases of treated samples (exposed to 100 ng/ml GA for 72 h) was superimposable to that of control cells. Taken together, these results suggest a direct effect of GA and 17-AAG on telomerase and, in accordance with recent data by Chang et al. (39), who demonstrated that antisense-mediated inhibition of HSP90 significantly decreased telomerase activity in HL60 leukaemia cells, further support the inference that this molecular chaperone plays a role in the assembly of the active holoenzyme.

An additional possible reason for telomerase inhibition after exposure of cells to ansamycin antibiotics is related to the concept that Akt, an HSP90-client protein (40), is involved in telomerase activation through the phosphorylation of its catalytic component hTERT (41). As a consequence, the possible inhibition of Akt protein expression after drug exposure (which has already been demonstrated in 17-AAG-treated colon cancer cell lines) (42) could lead to a decreased extent of hTERT phosphorylation and contribute to attenuating the telomerase activity in JR8 melanoma cells.

When JR8 cells were chronically treated with GA or 17-AAG (60 ng/ml of drug once every 3 days for 21 days) we were unable to detect any reduction in telomere length compared with control cells. This finding is in accordance with our previous results indicating that in the JR8 cell system prolonged inhibition of telomerase activity by different approaches—including peptide nucleic acids and ribozymes targeting hTR (30,43) and cytotoxic drugs (44)—did not result in any telomere shortening. Similar results were also recently obtained by Gan et al. (45), who found that telomere maintenance was not delayed by complete telomerase inhibition (which was achieved through 3’-azido-deoxythymidine or antisense hTR exposure and maintained for several weeks) in telomerase-positive SKOV-3 human ovarian cancer cells.
Moreover, in JR8 cells chronically exposed to GA or 17-AAG, senescence-associated β-galactosidase activity, a surrogate marker of senescence (46), was comparable to that of control cells (data not shown).

Several lines of evidence suggest that telomerase might play a role in cellular resistance to apoptosis. Specifically, in PC12 human pheochromocytoma cells the inhibition of telomerase activity induced by 24-h exposure to the oligonucleotide TTAGGG or to 3,3'-diethyloxacarbocyanine was associated with increased susceptibility to apoptosis induced by different stimuli, such as staurosporine, amyloid β-peptide and oxidative insult. Moreover, caspase inhibitors protected PC12 cells against the pro-apoptotic action of telomerase inhibitors, thus suggesting a site of action of telomerase prior to caspase activation (47). It has also been reported that stable overexpression of Bcl-2 in HeLa human cervical carcinoma cells resulted in increased telomerase activity and resistance to apoptosis (48). Again, several studies have demonstrated that down-regulation of telomerase by antisense oligonucleotides and ribozymes targeting hTR- or hTERT-induced apoptosis within a few days of treatment (30,49,50). The results of these studies cannot be explained by the classical model that predicts that telomerase inhibition has to be maintained for a certain number of rounds of cell divisions before it results in cell growth arrest as a consequence of telomere shortening (51). Conversely, they support a second possible mechanism which does not require telomere shortening and is probably related to the interference of inhibitors with the capping functions of telomerase to protect the telomere from signalling into cell-cycle arrest/apoptosis pathways (24).

Considering that apoptosis is an important mode of cell death induced by several anticancer drugs including 17-AAG (41), the role of telomerase in determining the chemosensitivity profile of tumour cells can be hypothesized. In the present study we demonstrated that the inherent level of telomerase activity influences the in vitro response of JR8 melanoma cells to 17-AAG. Specifically, the JR8-derived clones JR8pRcRzB2 and JR8pRcRzB15, which endogenously express a hammerhead ribozyme targeting hTR and are characterized by a markedly lower level of the enzyme’s catalytic activity than the parental cells (29), showed a significantly increased sensitivity to 17-AAG as a consequence of a significantly greater susceptibility to 17-AAG-induced apoptosis. Such results do not seem to be the reflection of general chemosensitization due to telomerase down-regulation in JR8pRcRzB2 and JR8pRcRzB15 clones, as we demonstrated previously that they do not differ from JR8 cells in their sensitivity profiles to a variety of anticancer agents including platinum compounds, taxanes and topoisomerase I inhibitors (29). The results of the present study corroborated previous findings by Incles et al. (35), who demonstrated that transfection of SKOV-1 human ovarian adenocarcinoma cells with an hTERT dominant negative human pheochromocytoma cells the inhibition of telomerase activity induced by 24-h exposure to the oligonucleotide TTAGGG or to 3,3'-diethyloxacarbocyanine was associated with increased susceptibility to apoptosis induced by different stimuli, such as staurosporine, amyloid β-peptide and oxidative insult. Moreover, caspase inhibitors protected PC12 cells against the pro-apoptotic action of telomerase inhibitors, thus suggesting a site of action of telomerase prior to caspase activation (47). It has also been reported that stable overexpression of Bcl-2 in HeLa human cervical carcinoma cells resulted in increased telomerase activity and resistance to apoptosis (48). Again, several studies have demonstrated that down-regulation of telomerase by antisense oligonucleotides and ribozymes targeting hTR- or hTERT-induced apoptosis within a few days of treatment (30,49,50). The results of these studies cannot be explained by the classical model that predicts that telomerase inhibition has to be maintained for a certain number of rounds of cell divisions before it results in cell growth arrest as a consequence of telomere shortening (51). Conversely, they support a second possible mechanism which does not require telomere shortening and is probably related to the interference of inhibitors with the capping functions of telomerase to protect the telomere from signalling into cell-cycle arrest/apoptosis pathways (24).

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mutant led to a 4-fold increased sensitivity to 17-AAG treatment with respect to parental cells, and suggest that the level of telomerase activity might be a determinant of the cellular response to 17-AAG.

However, it should be noted that there was no direct evidence that 17-AAG-induced apoptosis was the consequence of acute inhibition of telomerase in our melanoma cells. In fact, 17-AAG has been shown to elicit extremely widespread effects in cancer cells, including the inhibition of several protein kinases (Akt, c-Raf1, Erk, Src and others) that mediate survival signaling, thus inducing growth arrest and apoptosis in a variety of tumor models (17,42,52). Specifically, 17-AAG exposure has been demonstrated to induce mitochondrial release and cytosolic accumulation of cytochrome c and Smac/Diablo and to down-regulate XIAP and survivin proteins, thus resulting in the activation of caspase-9 and -3 (53).

In summary, we demonstrated in this study that telomerase is a target of 17-AAG and GA action and its inhibition may contribute to the overall cytotoxic activity of these drugs. Moreover, the finding that 17-AAG is more effective in tumour cells overexpressing the 17AAG target of 17-AAG and GA action and its inhibition may contribute to the overall cytotoxic activity of these drugs. Moreover, the finding that 17-AAG is more effective in tumour cells expressing low levels of telomerase activity suggests the opportunity to design combined treatments including 17-AAG and telomerase inhibitors and to test their antitumour potential.

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