Cell Death and Differentiation (2005) 12, 1429–1438
© 2005 Nature Publishing Group All rights reserved 1350-9047/05 $30.00
www.nature.com/cdd

Involvement of hTERT in apoptosis induced by interference with Bcl-2 expression and function

D Del Bufalo[1], A Rizzo[1], D Trisciuoglio[1], G Cardinali[2], MR Torrisi[3], U Zangemeister-Wittke[4], G Zupi[1] and A Biroccio*,[1]

[1] Experimental Chemotherapy Laboratory, 'Experimental Research Center', Regina Elena Cancer Institute, Rome 00158, Italy
[2] Cellular and Molecular Biology Laboratory 'San Gallicano' Dermatological Institute, Rome 00144, Italy
[3] Department of Experimental Medicine and Pathology, University of Rome 'La Sapienza', Rome 00161, Italy
[4] Department of Oncology, University Hospital Zurich, Zurich 8044, Switzerland
* Corresponding author: Dr A Biroccio, Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy.Tel: +39-06-52662569; Fax: +39-06-52662592; E-mail: biroccio@ifo.it

Received 11.11.04; revised 24.3.05; accepted 19.4.05; published online 27.5.05
Edited by A Villunger

Abstract

Here, we investigated the role of telomerase on Bcl-2-dependent apoptosis. To this end, the 4625 Bcl-2/Bcl-x_L bispecific antisense oligonucleotide and the HA14-1 Bcl-2 inhibitor were used. We found that apoptosis induced by 4625 oligonucleotide was associated with decreased Bcl-2 protein expression and telomerase activity, while HA14-1 triggered apoptosis without affecting both Bcl-2 and telomerase levels. Interestingly, HA14-1 treatment resulted in a profound change from predominantly nuclear to a predominantly cytoplasmic localization of hTERT. Downregulation of endogenous hTERT protein by RNA interference markedly increased apoptosis induced by both 4625 and HA14-1, while overexpression of wild-type hTERT blocked Bcl-2-dependent apoptosis in a p53-independent manner. Catalytically and biologically inactive hTERT mutants showed a similar behavior as the wild-type form, indicating that hTERT inhibited the 4625 and HA14-1-induced apoptosis regardless of telomerase activity and its ability to lengthening telomeres. Finally, hTERT overexpression abrogated 4625 and HA14-1-induced mitochondrial dysfunction and nuclear translocation of hTERT. In conclusion, our results demonstrate that hTERT is involved in mitochondrial apoptosis induced by targeted inhibition of Bcl-2.
Cell Death and Differentiation (2005) **12,** 1429–1438. doi:10.1038/sj.cdd.4401670; published online 27 May 2005

Keywords: telomerase; bcl-2; apoptosis; mitochondria

Abbreviations: hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase; hTERT-HA, hemagglutinin-tagged human telomerase reverse transcriptase; DN-hTERT, dominant-negative hTERT; ADR, adriamycin; sihTERT, RNA interference against hTERT; HA14-1, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate; DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; pAb, polyclonal antibody; PCR, polymerase chain reaction; TRAP, telomeric repeat amplification protocol; ITAS, internal TRAP assay standard; PI, propidium iodide; $\Delta\psi_m$, mitochondrial membrane potential; ROS, reactive oxygen species; DHE, dihydroethidium; CPT, camptothecin

Introduction

Telomerase, the ribonucleoprotein complex that adds telomeric repeats to the ends of chromosomes, is responsible for telomere maintenance,[1] and its expression is associated with cell immortalization and tumorigenesis.[2] The human telomerase holoenzyme consists of the RNA template subunit, human telomerase RNA (hTR),[3] the catalytic subunit, human telomerase reverse transcriptase (hTERT)[4] and several associated proteins involved in the regulation of telomerase activity and telomere length.[5] Introduction of hTERT into normal cells induces telomerase activation, telomere elongation and extension of life span,[2,6] suggesting a pivotal role for telomerase in preventing cellular senescence.

It has been also demonstrated that TERT may promote an immortal phenotype by preventing apoptosis.[7–10] Inhibition of telomerase in tumor cells by targeting hTR or hTERT usually results in telomere erosion and apoptosis after a certain number of cell divisions that are dependent on the initial telomere length.[9,11] Inhibition of hTERT by antisense strategy can also induce short-term apoptosis, without affecting telomere length.[12,13] These results are consistent with recent findings indicating that telomerase plays a crucial role in the protection of telomere capping and that inhibition of telomerase affects cell survival through pathways that are not dependent on telomere erosion.[14]

The relationship between telomerase and apoptosis has also been studied in response to DNA damage or DNA-damage-related signals that trigger cell death. Antisense inhibition of telomerase increases the susceptibility of glioblastoma cells to cisplatin-induced apoptosis[15] and enhances cell death in pheochromocytoma cells induced by a variety of stimuli.[7] Similarly, telomerase inhibition by ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to topoisomerase inhibitors through the activation of the apoptotic program.[16] On the other hand, cells with high levels of telomerase exhibit resistance to cell death following exposure to radiation.[17] Moreover, hTERT protects cultured neural cells from death induced by camptothecin (CPT)[18] and human lung fibroblasts against oxidative stress-induced apoptosis.[19]

In many cell types, apoptosis occurs through a sequence of events involving a number of gene products including the survival factor Bcl-2. Bcl-2 is an oncogene frequently deregulated in human cancers[20] and its overexpression has been shown to suppress apoptosis in response to different stimuli.[20–24] Moreover, Bcl-2, like other oncoproteins, has

been implicated in the regulation of telomerase activity.[25] The finding that enforced overexpression of Bcl-2 in tumor cells with low basal expression levels was accompanied by increased telomerase activity,[25] suggests a close linkage between telomerase and Bcl-2-mediated antiapoptosis. Evidence for a correlation among Bcl-2, telomerase and apoptosis was reported, but data are controversial and seem to be cell type dependent. Specifically, whereas IL-2 deprivation of IL-2-dependent cytotoxic T cells decreased Bcl-2 expression and telomerase activity without induction of apoptosis,[25] apoptosis induced by methionine aminopeptidase-2 inhibition in mesothelioma cells was associated with decreased telomerase activity and Bcl-2 downregulation.[26]

This study was designed to help clarifying the intriguing role of telomerase in Bcl-2-dependent apoptosis.

Results

4625 oligonucleotide and HA14-1 induce apoptosis in MCF7 ADR breast carcinoma cells

To induce Bcl-2-dependent apoptosis, the 4625 Bcl-2/Bcl-x_L bispecific antisense oligonucleotide and the Bcl-2 inhibitor ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14-1) were used.[27–30] MCF7 cells resistant to adriamycin (ADR) were treated with different concentrations of either 4625 or HA14-1 and the effects of both treatments on cell proliferation and apoptosis evaluated (Figure 1).

Panels a and b of Figure 1 show the number of cells and the analysis of apoptosis upon treatment with 0.3 and 0.6 μM 4625 oligonucleotide. As control, 4626 scramble oligonucleotide was used at the higher concentration. It is evident that 4625 induced a dose- and time-dependent inhibition of cell proliferation (Figure 1a). Compared with untreated or 4626-treated cells, 0.3 μM 4625 caused a reduction in cell number of about 25% at 48 h after the end of treatment. At 0.6 μM cell proliferation decreased by 50%. Cytofluorimetric analysis of the annexin V *versus* propidium iodide (PI) staining (Figure 1b) revealed that 4625 induced apoptosis (annexin V^+/PI^- region of the dot plot panels). Following treatment with 0.3 and 0.6 μM 4625, the percentage of annexin V^+/PI^- cells increased with dose and time after the end of treatment. Upon incubation with 0.3 μM, 28% of apoptotic cells were found 48 h after the end of treatment, and the number of apoptotic cells increased to 45% upon treatment with 0.6 μM. No significant increase in the number of apoptotic cells was observed upon treatment with the 4626 control sequence.

Panels c and d of Figure 1 show the results obtained upon treatment with 10, 20 and 40 μM HA14-1 for 16 and 24 h. Compared with untreated cells, while no inhibition of cell proliferation was observed following exposure of cells to 10 μM HA14-1, the number of cells significantly decreased upon treatment with doses of 20 and 40 μM (Figure 1c). In

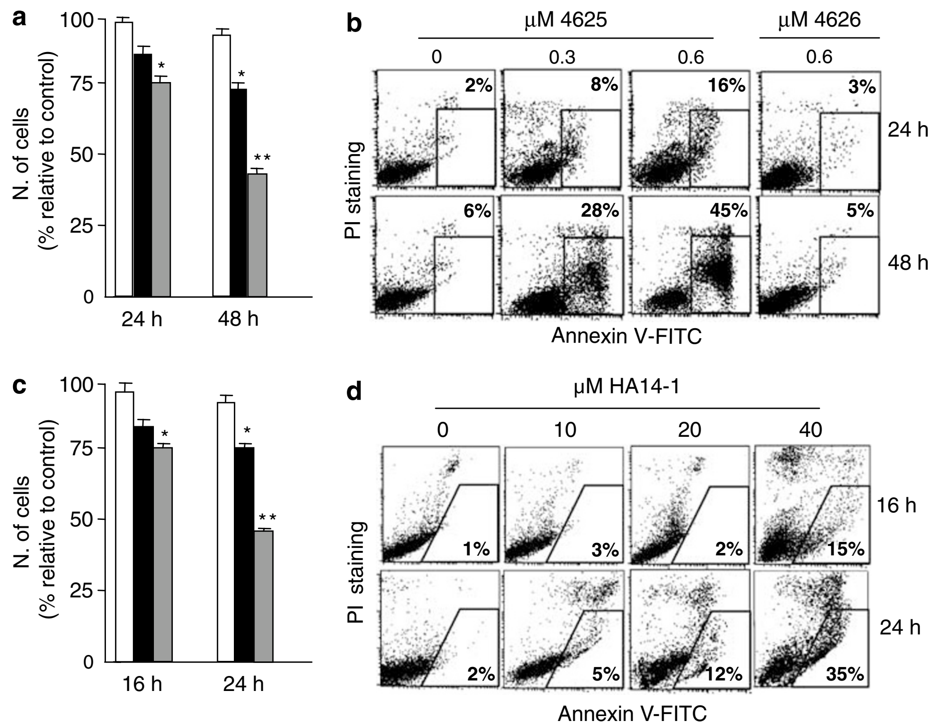


Figure 1 4625 oligonucleotide and HA14-1 induce apoptosis. Proliferation assay (**a**) and cytofluorimetric analysis of annexin V *versus* PI staining (**b**) in MCF7 ADR cells 24 and 48 h after the end of treatment with 4625 at two different concentrations: 0.3 (black columns) and 0.6 μM (gray columns). 4626 (white columns) represents oligonucleotide control. Proliferation assay (**c**) and cytofluorimetric analysis of the annexin V *versus* PI staining (**d**) in MCF7 ADR cells treated for 16 and 24 h with HA14-1 at 10 (white columns), 20 (black columns) and 40 μM (gray columns) concentrations. The percentage shown in the annexin V^+/PI^- region of each histogram represents the fraction of apoptotic cells. Each panel is representative of four separate experiments with comparable results. Statistical significance: *P*-values are (*) $P<0.05$ and (**) $P<0.01$ for treated *versus* untreated cells

particular, at 40 μM the inhibition of cell proliferation was about 25 and 60% upon a 16 and 24 h treatment, respectively. Cytofluorimetric analysis of apoptosis revealed that HA14-1 added to cells for 24 h induced apoptosis at the two highest doses (Figure 1d). Compared with untreated cells, 12 and 35% of annexin V^{+}/PI^{-} cells were observed following a 24 h treatment with 20 and 40 μM HA14-1, respectively.

4625 oligonucleotide and HA14-1 modulate telomerase activity

Since Bcl-2 has been implicated in the regulation of telomerase,[25] we investigated the effect of 4625 oligonucleotide and HA14-1 on Bcl-2 expression and telomerase activity. As shown in Figure 2a, treatment with 4625 efficiently downregulated the Bcl-2 protein in a dose-dependent manner. Bcl-2 expression was completely blocked after treatment with 0.6 μM 4625. Despite the potential bispecificity of the antisense, under the transfection conditions chosen for our experiments no change in Bcl-x_L expression was observed. Telomeric repeat amplification protocol (TRAP) assay (Figure 2b) revealed that, compared with untreated cells, telomerase activity was inhibited in a dose-dependent manner following treatment with 4625, while it was not affected by the 4626 control sequence. Internal TRAP assay standard (ITAS) was amplified to the same extent in all samples, excluding the presence of *Taq* polymerase inhibitors in 4625-treated cells. On the contrary, as expected, HA14-1 did not modulate Bcl-2 protein levels (Figure 2c). Similarly, telomerase activity was unchanged following HA14-1 treatment at all doses tested (Figure 2d).

Since nuclear localization of TERT seems to be associated with telomerase function,[31,32] we evaluated whether HA14-1 interfered with hTERT distribution. Analysis of telomerase activity in nuclear and cytosolic extracts demonstrated that HA14-1 treatment markedly reduced nuclear hTERT activity, whereas the hTERT activity increased in the cytosolic fraction (Figure 3A). Immunohistochemical analysis confirmed these findings and demonstrated that while in untreated cells hTERT was exclusively localized in the nucleus, HA14-1

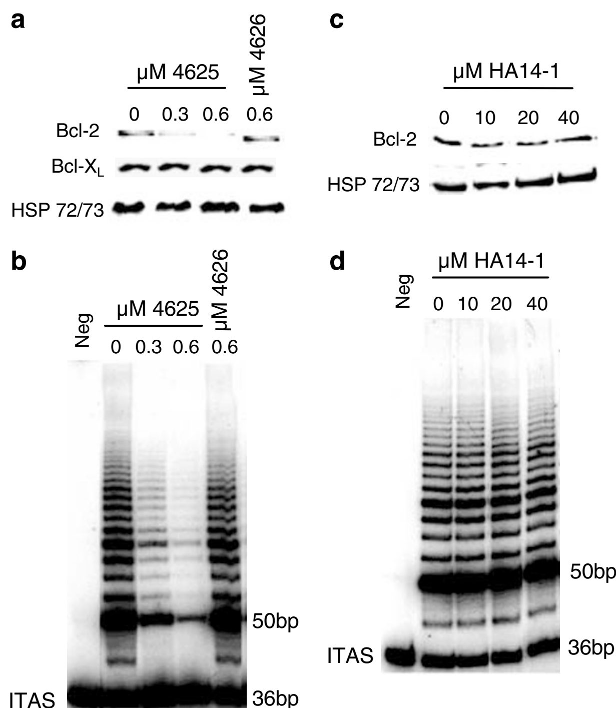
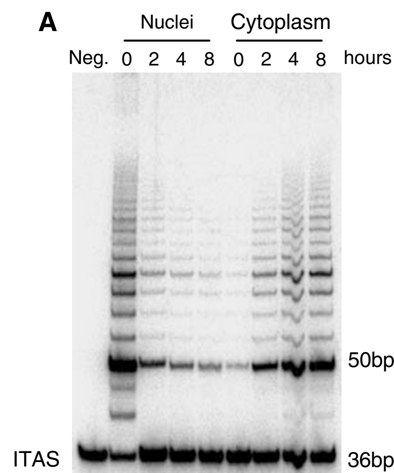


Figure 2 4625 oligonucleotide, but not HA14-1, decreases Bcl-2 protein expression and telomerase activity. Western blot analysis of Bcl-2 and Bcl-x_L protein expression (**a**) and TRAP gel (**b**) in MCF7 ADR cells 24 h after the end of treatment with 0.3 and 0.6 μM 4625 oligonucleotide. 4626 represents the control oligonucleotide. Western blot analysis of Bcl-2 (**c**) and TRAP gel (**d**) in MCF7 ADR cells treated for 16 h with 10, 20 and 40 μM HA14-1. TRAP assay was performed using total protein extracts. Each panel is representative of three separate experiments with comparable results



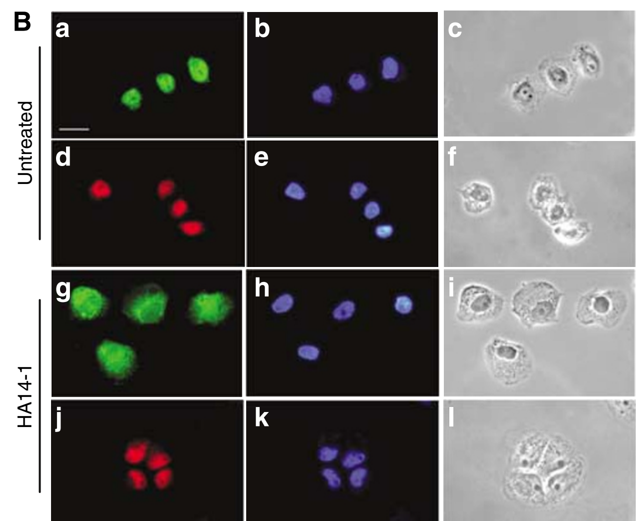


Figure 3 HA14-1 treatment induces hTERT translocation from the nucleus to the cytosol. (**A**) TRAP assay performed using nuclear and cytosolic protein extracts in MCF7 ADR cells untreated (0) and treated for 2, 4, and 8 h with 40 μM HA14-1. (**B**) Immunofluorescence analysis of hTERT protein localization in MCF7 ADR cells untreated and treated for 8 h with 40 μM HA14-1. The p53 protein was used as control. Immunolabeling with anti-TERT (a, g) and p53 (d, j), parallel staining with DAPI (b, e, h, k), phase contrast microscopy (c, f, i, l). Bar, 20 μm. Each panel is representative of three separate experiments with comparable results

induced a profound change in the hTERT staining pattern, due to its localization not only in the nucleus but also in the cytosol. Nuclear control staining of p53 was not affected by the treatment (Figure 3B).

Overexpression of hTERT protects from HA14-1 and 4625-induced apoptosis independently of its enzymatic and telomere-maintaining activity

To define the role of hTERT in apoptosis induced by 4625 and HA14-1, Bcl-2-overexpressing cells and cells expressing different hTERT variants were used. Moreover, endogenous hTERT protein was downregulated using specific siRNA. Figure 4 shows Bcl-2/hTERT protein expression and telomerase activity in the different experimental models. The Bcl-2 overexpressing clones MAB27 and MAB30[33] showed an about three-fold increase in hTERT protein (Figure 4a) and enhanced telomerase activity respect to MAN9 control clone and MCF7 ADR parental cells (Figure 4b). Compared with the controls, hTERT increased in cells infected with the different hTERT variants, while it was markedly decreased in RNA interference against hTERT (sihTERT)-treated cells. Moreover, Bcl-2 expression did not change regardless of the type of hTERT protein present in the cells (Figure 4c). Both wild-type and hemagglutinin-tagged human telomerase reverse transcriptase (hTERT-HA)-transfected cells showed elevated telomerase activity as compared to cells infected with the control virus. On the contrary, a marked decrease of hTERT activity was found in both dominant-negative hTERT (DN-hTERT) and in sihTERT-expressing cells (Figure 4d).

Figure 5 shows cytofluorimetric analysis of annexin V *versus* PI staining performed after treatment with both 4625 oligonucleotide and HA14-1 in control cells and in cells overexpressing either the different hTERT variants or Bcl-2 or treated with hTERT siRNA. It is evident that overexpression of wild-type hTERT protected from apoptosis induced by both 4625 oligonucleotide and HA14-1 treatment: the percentage of annexin V^+/PI^- cells decreased from 39 to 15%, and from 38 to 11% respectively. Similarly, Bcl-2 overexpressing cells were resistant to both 4625 and HA14-1-induced apoptosis. Interestingly, both the catalytically inactive DN-hTERT and the biologically inactive hTERT-HA mutant showed a similar

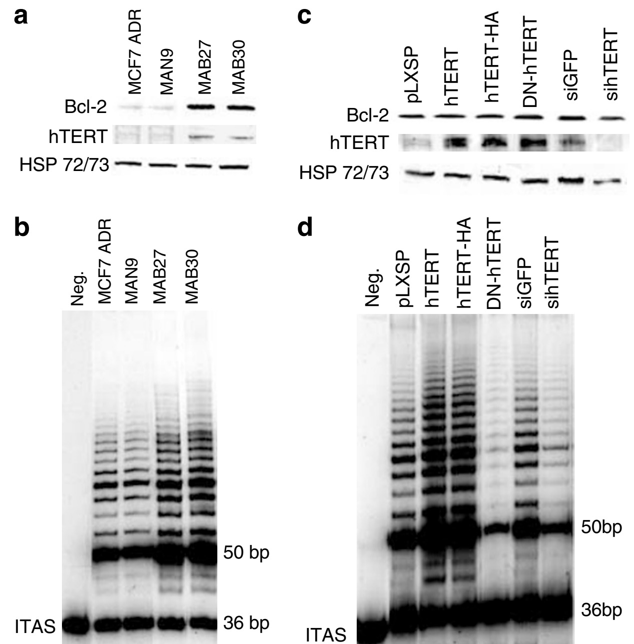


Figure 4 Modulation of Bcl-2 and hTERT interferes with telomerase activity. Western blot analysis of Bcl-2 and hTERT protein expression (**a**) and analysis of telomerase activity (**b**) in MCF7 ADR cells, MAN9 control clone and two Bcl-2 overexpressing clones (MAB27 and MAB30). Western blot analysis of Bcl-2 and hTERT protein expression (**c**) and analysis of telomerase activity (**d**) performed in the following cells: control virus-infected cells (pLXSP); cells infected with hTERT, hTERT-HA and DN-hTERT retroviruses; cells transfected with control (siGFP) and hTERT interference RNA (sihTERT). Each panel is representative of four separate experiments with comparable results

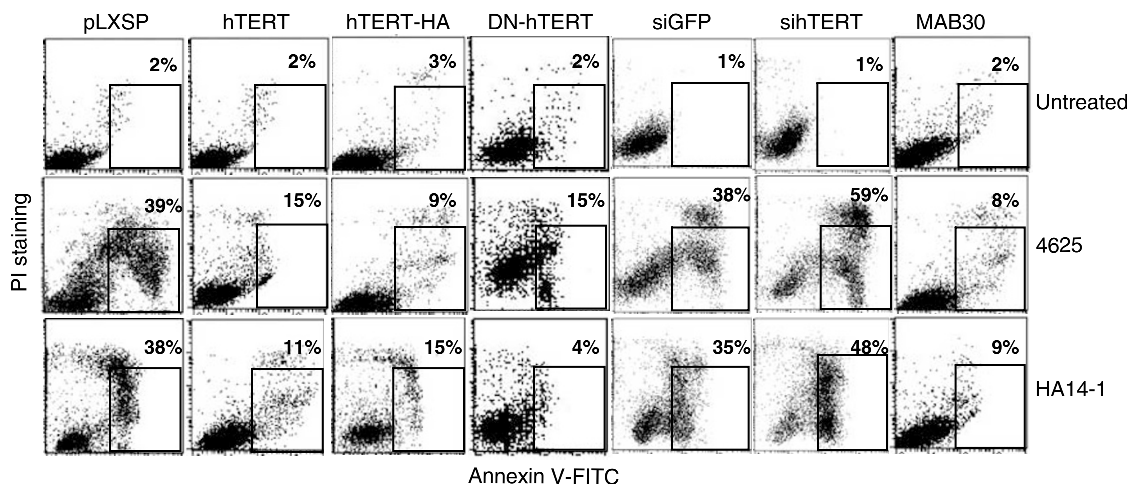


Figure 5 hTERT interferes with 4625 and HA14-1-induced apoptosis independently of its catalytic activity and *in vivo* effect on telomeres. Cytofluorimetric analysis of the annexin V *versus* PI staining in cells untreated and treated with 4625 oligonucleotide (0.6 μM, 48 h) or HA14-1 (40 μM, 24 h). The following cells were used: control virus-infected cells (pLXSP); cells infected with hTERT, hTERT-HA and DN-hTERT retroviruses; MAB30 Bcl-2 overexpressing cells; cells transfected with GFP-specific control siRNA (siGFP) and hTERT-specific siRNA (sihTERT). Each panel is representative of three separate experiments with comparable results

behavior as the wild-type form, indicating that hTERT protects from Bcl-2-dependent apoptosis regardless of telomerase activity and its ability to telomere lengthening. Reduction of endogenous hTERT protein by RNA interference markedly increased Bcl-2-dependent apoptosis and thus confirmed the antiapoptotic function of hTERT.

To further corroborate this finding, we investigated the effect of hTERT on apoptosis induced by 4625 or HA14-1 in additional tumor cell lines, and assessed the role of hTERT in response to CPT, a standard anticancer agent known to induce apoptosis. As shown in Figure 6a, both 4625 and HA14-1 efficiently induced apoptosis in M14 human melanoma cells infected with control virus. Overexpression of hTERT protected from Bcl-2-dependent apoptosis and the percentage of annexin V^+/PI^- cells decreased from 30% to less than 10% upon hTERT upregulation. Moreover, CPT also triggered apoptosis in M14 cells and overexpression of hTERT, even though less efficiently, antagonized this drug-induced cell death.

Since overexpression of hTERT may modulate p53 activity,[34] we investigated the role of p53 in the ability of hTERT to protect from Bcl-2-dependent apoptosis. HCT116 p53 $+/+$ human colon carcinoma cells and its isogenic p53 knockout variant HCT116 p53 $-/-$ were infected with either control or hTERT retrovirus, and the effect of HA14-1 was evaluated. Figure 6b shows that HA14-1 induced apoptosis both in parental HCT116 and p53 $-/-$ variant cells, even though the absence of p53 marginally inhibited HA14-1-induced cell death. However, overexpression of hTERT antagonized apoptosis triggered by HA14-1 both in p53 wild-type and knockout cells.

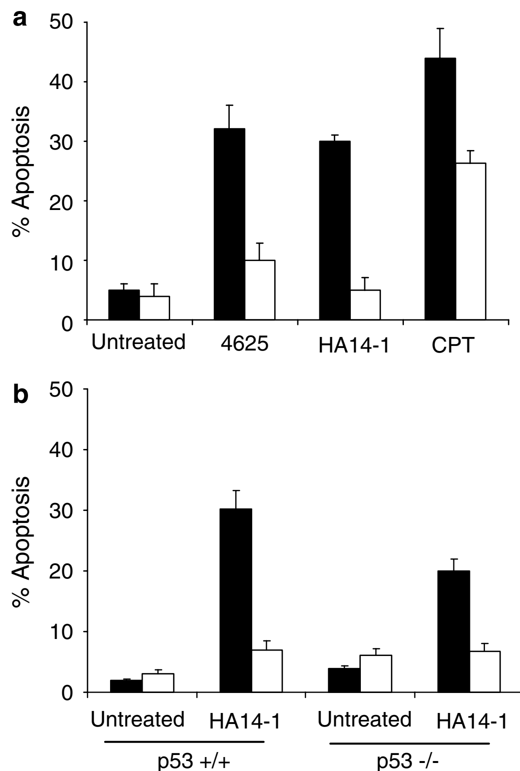


Figure 6 Protection from apoptosis by hTERT is independent of p53. (**a**) Percentage of apoptotic cells calculated by cytofluorimetric analysis of annexin V *versus* PI staining in M14 melanoma cells untreated or treated with 4625 (0.6 μM, 48 h), HA14-1 (40 μM, 24 h) and CPT (0.5 μM, 48 h). Black bars, cells infected with control virus; white bars, cells infected with hTERT retrovirus. (**b**) Percentage of apoptotic cells in HCT116 colon carcinoma line (p53 $+/+$) and its isogenic p53 knockout variant (p53 $-/-$) untreated or treated with HA14-1 (40 μM, 24 h). Black bars, cells infected with control virus; white bars, cells infected with hTERT retrovirus

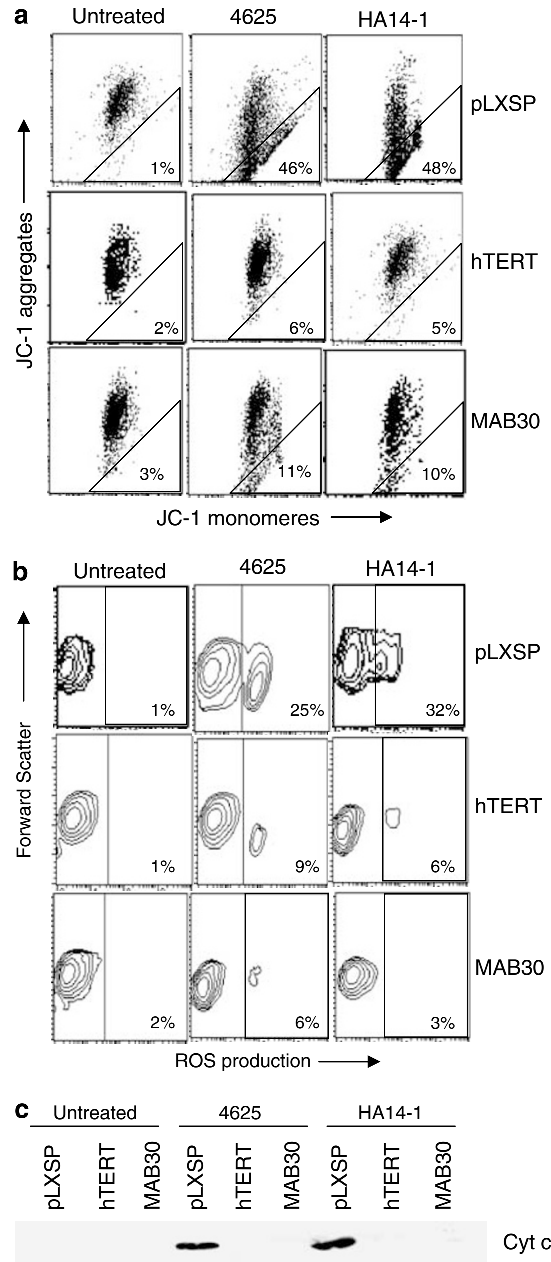


Figure 7 Bcl-2 and hTERT overexpression prevent 4625 and HA14-1-induced mitochondrial dysfunction. Analysis of $\Delta\psi_m$ (**a**), ROS production (**b**) and cytochrome c (cytc) release (**c**) in control (pLXSP), hTERT and Bcl-2 overexpressing cells (MAB30) untreated or treated with 4625 (0.6 μM, 24 h) or HA14-1 (40 μM, 8 h). Each panel is representative of three separate experiments with comparable results

Overexpression of hTERT prevents mitochondrial dysfunction and nuclear export of hTERT protein

Since mitochondrial alterations play a pivotal role in Bcl-2-dependent apoptosis, we determined whether hTERT exerts its antiapoptotic action at a specific step of the cell death pathway. Figure 7 shows the mitochondrial membrane potential ($\Delta\psi_m$), reactive oxygen species (ROS) production and cytochrome *c* release after treatment with 4625 oligonucleotide and HA14-1 in control cells and cells overexpressing either hTERT or Bcl-2. A significant reduction in $\Delta\psi_m$ was observed after treatment of control virus-infected cells with either of the treatments (Figure 7a). On the contrary, no reduction of $\Delta\psi_m$ was seen in the 4625- and HA14-1-treated hTERT or Bcl-2 overexpressing cells. Similarly, the generation of ROS (Figure 7b) and release of cytochrome *c* (Figure 7c) was observed only in control virus-infected cells treated with either of the agents.

Finally, we investigated the effect of hTERT or Bcl-2 overexpression on hTERT localization in untreated and HA14-1-treated cells. Figure 8 shows that hTERT and Bcl-2 overexpression did not modify hTERT nuclear localization. Interestingly, while HA14-1 induced nuclear export of hTERT, the signal remained confined to the nucleus both in hTERT and Bcl-2 overexpressing cells treated with HA14-1. Quantitative fluorescence analysis revealed an increase in the signal intensity in the cytoplasm of HA14-1-treated control cells (28.70 ± 5.6) compared to the cytosolic values obtained in pLXSP untreated cells (3.30 ± 2.2) or in HA14-1-treated hTERT (3.25 ± 1.92) and Bcl-2-overexpressing cells (2.25 ± 2.2). These findings were confirmed by confocal microscopy (Figure 9), which also demonstrated that the cytosolic hTERT protein fraction in HA14-1-treated cells localized with the mitochondria. On the contrary, nuclear hTERT was retained in the nucleus and did not colocalize with the mitochondrial marker. Inhibition of nuclear export using

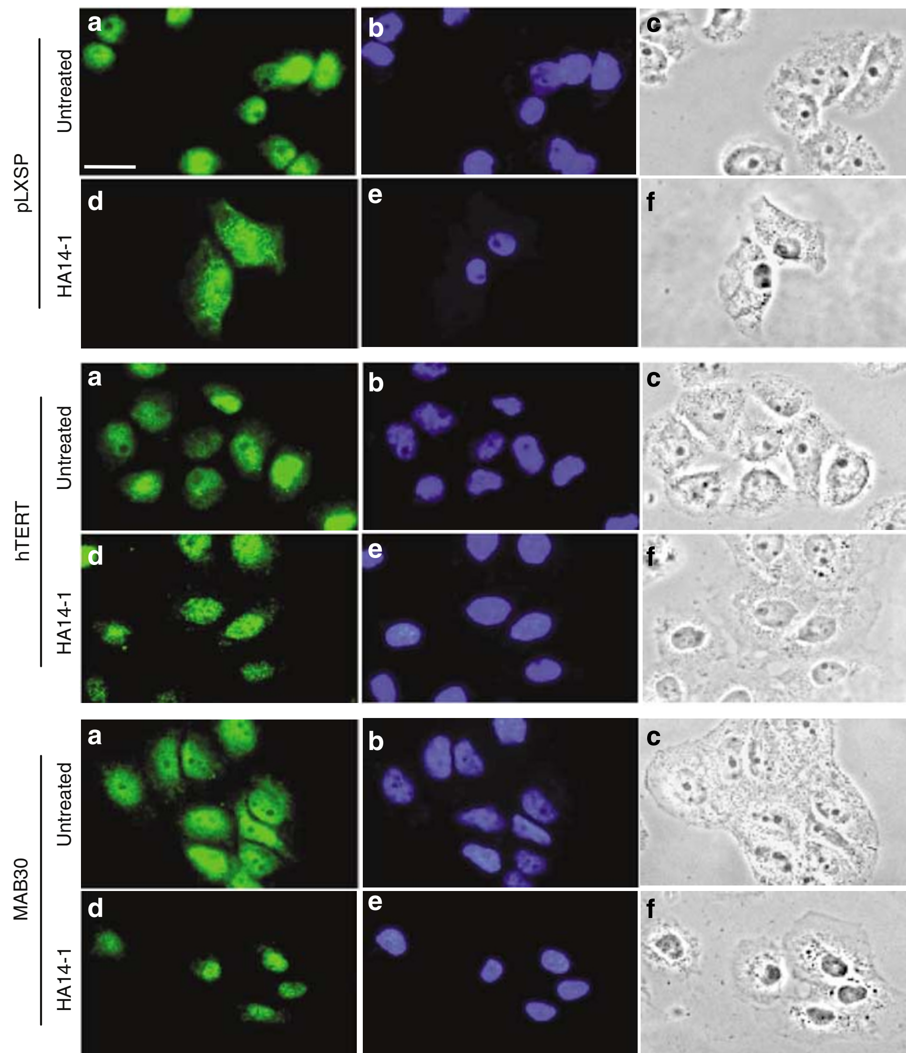


Figure 8 Bcl-2 and hTERT overexpression block HA14-1-induced nuclear export of hTERT. Immunofluorescence analysis of hTERT protein localization in control (pLXSP), hTERT and Bcl-2 overexpressing cells (MAB30) untreated and treated with HA14-1 (40 μM for 8 h). Immunolabeling with anti-hTERT (**a**, **d**), parallel staining with DAPI (**b**, **e**) and phase contrast microscopy (**c**, **f**). Bar, 20 μm. Each panel is representative of three separate experiments with comparable results

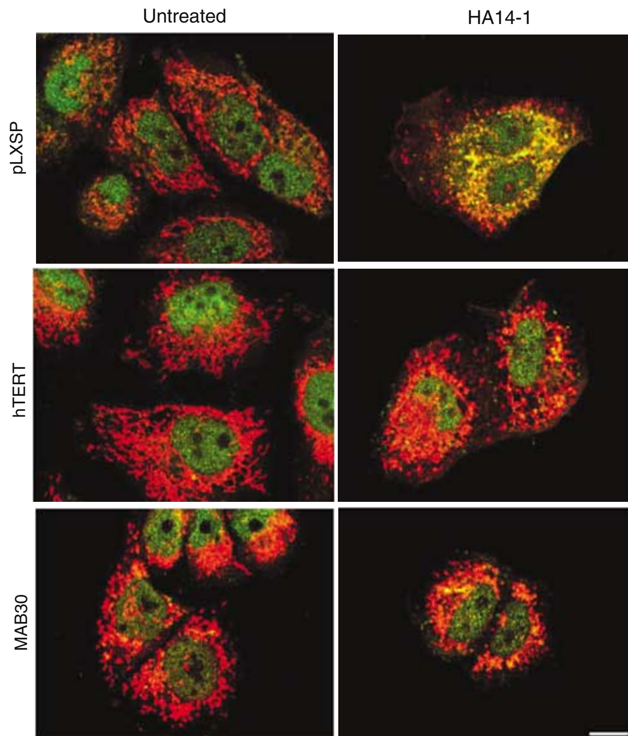


Figure 9 hTERT localizes to mitochondria in HA14-1-treated cells. Immunofluorescence of hTERT protein localization using anti-hTERT antibody in control cells (pLXSP), hTERT and Bcl-2-overexpressing cells (MAB30) untreated or treated with HA14-1 (40 μM for 8 h). Confocal microscopy analysis of HA14-1-treated control cells reveals the presence of hTERT in cytoplasmic dots positive for HSP60, a marker for mitochondria, and the extent of colocalization of the two signals (hTERT: green; HSP60: red) is shown in yellow. Bar, 10 μm

Leptomycin B, partially protected from HA14-1-induced cell death as demonstrated by 35 and 20% apoptosis in untreated and treated cells, respectively. Altogether our data indicate that hTERT translocation plays a role in Bcl-2-dependent apoptosis.

Discussion

In this study, we investigated the role of telomerase on apoptosis induced by modulation of Bcl-2 expression and function using the 4625 Bcl-2/Bcl-x_L bispecific antisense oligonucleotide[28,29] and the HA14-1 Bcl-2 inhibitor.[27,30] In MCF7 ADR human breast carcinoma cells, we first evaluated the effect of the two Bcl-2-dependent apoptosis inducers on telomerase activity. 4625 decreased both Bcl-2 expression and telomerase activity in a dose-dependent manner and induced apoptosis. Conversely, HA14-1 triggered apoptosis without affecting Bcl-2 protein expression and telomerase activity. The data obtained with 4625 and HA14-1 suggest a strong link between telomerase activity and Bcl-2 expression rather than function. These findings are consistent with those of others demonstrating that Bcl-2 is implicated in the regulation of telomerase in different human cancer cells.[25] The mechanism involved in the regulation of Bcl-2-dependent telomerase activity remains to be investigated. Bcl-2 might modulate telomerase activity by regulating hTERT at the transcription or post-transcription level. Our previous data demonstrate that Bcl-2 overexpression increases the expression of NF-*k*B and ERK 1/2, two molecules involved in the regulation of hTERT.[35,36]

The Bcl-2 inhibitor HA14-1, although unable to inhibit the overall telomerase activity, altered intracellular hTERT protein localization, indicating that modulation of Bcl-2 function interferes with telomerase function by inducing nuclear export of hTERT. Recently, translocation of hTERT has been described as an additional mechanism for post-transcriptional regulation.[31,32] Specifically, hTERT was shown to translocate from the cytoplasm to the nucleus in T cells and smooth muscle cells upon stimulation with growth factors.[31,37] Another study demonstrated that 14-3-3 signaling proteins bind to hTERT thereby preventing nuclear export.[32] More recently it has been reported that increased generation of ROS promotes the export of hTERT from the nucleus into the cytosol through nuclear pores.[38,39] Nuclear export occurs through ROS-induced hTERT phosphorylation mediated by Src-kinases.[38] Since in our experiments ROS are generated by HA14-1 treatment, we can speculate that activation of Src-kinases could be involved in the observed nuclear export of hTERT.

Overexpression of Bcl-2 increased hTERT protein and telomerase activity, and protected cells from 4625 and HA14-1-induced apoptosis. To better define the role of telomerase on Bcl-2-dependent apoptosis, telomerase activity was either upregulated by infection with a retrovirus encoding hTERT or decreased by RNA interference. Our results demonstrate that 4625 and HA14-1-induced apoptosis was abrogated following hTERT overexpression and markedly increased by hTERT-specific siRNA treatment, indicating that hTERT was crucial for cell survival. This is in line with findings demonstrating that suppression of telomerase promotes apoptosis in neuronal cells, germ cells and thymocytes, whereas overexpression of hTERT prevents apoptosis in cardiac myocytes.[40–44] The ability of hTERT to antagonize Bcl-2-dependent apoptosis was corroborated using two additional tumor cell lines of different histotypes where it was found to be p53-independent, since both p53 wild-type parental and knockout variant cells were protected from cell death.

In this study, we also found that the catalytically inactive DN-hTERT and the biologically inactive hTERT-HA mutant showed a similar behavior as the wild-type form, suggesting that hTERT-mediated apoptosis protection does not require telomerase activity and *in vivo* effects on telomeres. Our findings together with those of others assign to hTERT a putative prosurvival and antiapoptotic role, which is independent of its enzymatic and telomere elongation activity. The antiapoptotic role of telomerase, which is distinct from its telomere-maintenance function, seems to be related to the ability of hTERT to enhance genomic stability and repair.[45] The antiapoptotic activity of hTERT was indeed described in experiments where cell death was induced by DNA damage or DNA-damage-related signals.[45] The data reported in this study demonstrate, for the first time, that telomerase attenuates mitochondrial apoptosis induced by targeted intervention in Bcl-2 expression and function. Moreover, we found that hTERT overexpression interferes at an early stage

of cell death pathway upstream of mitochondrial alterations. Therefore, even though we did not found any change in Bcl-2 expression after hTERT upregulation, our results suggest that hTERT may directly or indirectly modulates the expression of genes implicated in apoptosis. This is consistent with recent data demonstrating that telomerase not only stabilizes telomeres but also activates gene transcription.[46] Abrogation of HA14-1-induced apoptosis by hTERT overexpression was associated with inhibition of nuclear export of endogenous hTERT, and the finding that Leptomycin B protected from cell death indicates that nuclear export of hTERT is involved in Bcl-2-dependent apoptosis. This is in good agreement with recent data demonstrating that nuclear-targeted hTERT shows enhanced antiapoptotic activity in human embryonic kidney cells.[38] Altogether, this suggests that nuclear export of hTERT induced by HA14-1 and/or decreased expression of telomerase by 4625 contributes to Bcl-2-dependent apoptosis.

In conclusion, although the molecular basis of the anti-apoptotic function of telomerase remains to be investigated in more depth, to the best of our knowledge, we provide for the first time evidence that telomerase inhibits apoptosis induced by targeted intervention in Bcl-2 function and expression. Our findings suggest an additional mechanism of cytoprotection by hTERT, which is related to its ability to interfere with the intrinsic apoptosis pathway controlled by mitochondria.

Materials and Methods

Cell lines and culture conditions

MCF7 ADR human breast carcinoma cells resistant to ADR and the human melanoma line M14 were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FCS, 2 mM L-glutamine, antibiotics. The HCT116 human colon carcinoma line and its isogenic p53 knockout variant HCT116 p53−/− were maintained in complete D-MEM medium (Invitrogen). Two clones expressing high levels of exogenous Bcl-2 protein (MAB27 and MAB30) and a neo-transfected clone (MAN9) were previously obtained by transfecting the MCF7 ADR cells with a Bcl-2-expression vector or the native vector alone.[33] All transfectants were grown in complete medium containing neomycin (0.8 mg/ml, Invitrogen).

Viral infection and transfection experiments

Cells were infected overnight with retrovirus encoding for different variants of hTERT. Viruses were generated as previously described.[47] Briefly, the Phoenix amphotropic packaging line was transfected with the following retroviral plasmids: pLXSP/hTERT (carrying the wild-type hTERT cDNA), pLXSP/hTERT-HA (carrying the mutant hemagglutinin-tagged hTERT, which is catalytically active but unable to maintain telomeres[48]), pLXSP/DN-hTERT (carrying the dominant-negative catalytically inactive mutant of hTERT) or pLXSP (encoding only the gene for the puromycin resistance). Transfected cells were incubated for 48 h at 37°C for virus production and the virus-containing medium was collected, filtered (0.45 μm filter) to remove packaging cells and used to infect the target cells.

For silencing of hTERT, siRNA was synthesized by Dharmacon Research (Lafayette, CO, USA) using the sequence reported by Kosciolek *et al.*[49] Cells were exposed to 100 nM siRNA in the presence of Lipofectamine 2000 (Invitrogen) for 72 h. Control experiments were performed using GFP-specific siRNA (Dharmacon).

Targeted inhibition of Bcl-2

The 4625 Bcl-2/Bcl-x_L bispecific 2′-O-(methoxy)ethyl-modified gapmer antisense oligonucleotide is fully complementary to the Bcl-2 mRNA and has three mismatching nucleotides to the Bcl-x_L mRNA.[28] Oligonucleotide 4626 is scrambled sequence control to 4625. Oligonucleotides were delivered into cells in form of complexes with the transfection reagent lipofectin (Invitrogen) as previously described.[28] Cells were incubated with different concentrations of oligonucleotides (0.3 and 0.6 μM) and lipofectin for 6 h.

The small-molecule Bcl-2 inhibitor HA14-1 was purchased from Sigma Chemical Co. (St Louis, MO, USA). HA14-1 was freshly prepared by dissolving in dimethyl sulfoxide (DMSO) prior to dilution to appropriate concentrations. Cells were treated with different concentrations of HA14-1 ranging from 10 to 40 μM. Leptomycin B (Sigma Milan, Italy) was used at a concentration of 10 nM.

The topoisomerase inhibitor CPT was purchased from Sigma and used at the concentration of 0.5 μM. Drug dilutions were freshly prepared prior to each experiment. At the end of each treatment the medium was replaced with fresh growth medium and, at different time points, cells were harvested, counted and assayed for cell viability (Tripan blue dye exclusion).

Western blotting

Western blotting was performed as previously described.[50] Briefly, 40 μg of total proteins were loaded on denaturing SDS-PAGE. Immuno-detection of hTERT, Bcl-2 and Bcl-x_L proteins was performed respectively using rabbit anti-hTERT polyclonal antibody (pAb) (L-20 Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 100 dilution), mouse anti-Bcl-2mAb (124 Dako s.p.a. Milan, Italy; 1 : 200 dilution) and rabbit anti- Bcl-x_L (S18, Santa Cruz; 1 : 500 dilution). To control for the amount of proteins transferred to nitrocellulose membranes, HSP 72/73 was used as control and detected by an anti-human HSP 72/73 (Calbiochem Cambridge, MA, USA; 1 : 1000 dilution). The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad, Milan, Italy) and normalized to HSP 72/73.

Telomeric repeat amplification protocol

Telomerase enzyme activity was measured on total and fractionated protein extracts using the polymerase chain reaction (PCR)-based TRAP kit (Intergen Company, Oxford, UK). To obtain nuclear and cytosolic fractions, cells were incubated in cytosolic extraction buffer (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 μg/ml Aprotinin. 0.5 mM PMSF) for 15 min at 4°C. After addition of NP-40 and further incubation for 1 min at 4°C, samples were centrifuged at 14 000 $\times$ g for 5 min at 4°C. The resulting supernatant contained the cytosolic fraction. The pellet was resuspended in CHAPS lysis buffer (provided by Intergen) and processed according to the manufacturer's instructions. The purity of the nuclear and cytosolic fractions was ensured by immunoblotting with topoisomerase 1 (nuclear) and HSP 70 (cytosolic). 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 ITAS and each extract was tested for heat sensitivity. Each set of TRAP assay included a control reaction without extract.

Evaluation of apoptosis

Apoptosis was detected by flow cytometric analysis of annexin V staining. Annexin V-FITC *versus* PI assay (Vibrant apoptosis assay, V-13242, Molecular Pobes, Eugene, OR, USA) was performed as previously described.[51] Briefly, adherent cells were harvested and suspended in the annexin-binding buffer (1×10^6 cells/ml). Thereafter, cells were incubated with annexin V-FITC and PI for 15 min at room temperature in the dark and immediately analyzed by flow cytometry. The data are presented as bi-parametric dot plots showing annexin V-FITC green fluorescence *versus* PI red fluorescence.

Immunofluorescence analysis

Cells were plated on coverslips coated with 2% gelatin onto 24-well plates, fixed with 4% parafomaldehyde in PBS for 30 min at 25°C and permeabilized with 0.1% Triton X-100 in PBS for 5 min. For immunolabeling experiments, cells were incubated with the following primary antibodies: anti-hTERT rabbit pAb, (H-231, Santa Cruz Biotechnology; 1 : 20 dil.), anti-HSP60 mAb (H99020-050, BD Biosciences; 1 : 100 dil.) and anti-p53mAb (DO-1, Santa Cruz Biotechnology; 1 : 100 dil.). The primary antibodies were visualized using the following secondary antibodies: rabbit anti-goat IgG-FITC (Cappel Research Products, Durham, NC, USA; 1 : 300 dil.), rabbit anti-mouse IgG-Texas Red (Jackson Immuno Research Laboratories Inc., PA, USA; 1 : 200 dil.). Nuclei were visualized using DAPI (Sigma Chemicals; 1 : 10 000 dil.). Fluorescence signals were analyzed either by recording stained images using a CCD camera (Zeiss, Oberkochen, Germany) and IAS2000/H1 software (Delta Sistemi, Roma, Italy) or by confocal vertical (*x*–*z*) sections (interval: 0.5 μm) obtained with a Zeiss Confocal Laser Scan Microscope.

Quantitative analysis of the fluorescence intensity on the nuclear or the cytosolic areas of the cells was performed evaluating at least 100 cells for each condition, randomly taken from 10 different microscopic fields.

Detection of cytosolic cytochrome *c*

Cytochrome *c* release into the cytosol was detected as previously described.[51] Cells were harvested and washed with PBS, then collected by centrifugation at $700 \times g$ for 7 min at 4°C. The cell pellet was resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES–NaOH, pH 7.4, 50 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol and protease inhibitors. After 30 min incubation on ice, cells were homogenized using a glass Dounce homogenizer. Cell homogenates were spin at $14\,000 \times g$ for 15 min at 4°C and supernatants were removed and stored at −80°C until analysis by gel electrophoresis. In all, 20 μg of cytosolic protein extracts were run on denaturating 12% SDS-PAGE. Mouse anti-cytochrome *c* antibody (7H8.2c12, PharMingen, San Diego, CA, USA; 1 : 500 dilution) was used to detect protein expression in the extramitochondrial compartment. ECL was employed for chemoluminescence detection.

Mitochondrial membrane potential

$\Delta\psi_m$ was assessed using the JC-1 dye as previously described.[52] Briefly, adherent cells (about 5×10^5) were first assayed for viability and then loaded with 10 μM JC-1 in medium for 30 min at 37°C in the dark. After incubation, cells were washed twice, resuspended in PBS and immediately analyzed by flow cytometry. The data are presented as bi-parametric panels with the green J-monomers fluorescence plotted *versus* the red J-aggregates fluorescence. Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Production of ROS

ROS production was measured as previously described.[50] Briefly, adherent cells (about 5×10^5) were first assayed for viability and then incubated with 4 μM dihydroethidium (DHE, Molecular Probes) for 45 min at 37°C in PBS. After incubation, cells were immediately analyzed by flow cytometry. The data are presented as bi-parametric panels with the red DHE fluorescence intensity plotted *versus* the forward scatter.

Statistical analysis

The results are presented as means $\pm$ S.D. Significant changes were assessed using Student's *t*-test for unpaired data, and *P*-values < 0.05 were considered significant.

Acknowledgements

This work was supported by grants from the Italian Association for Cancer Research (AIRC), Ministero della Sanità, and CNR-MIUR. D. Trisciuoglio and A. Rizzo are recipients of a fellowship from Italian Foundation for Cancer Research (F.I.R.C.).

We thank Dr Bert Vogelstein for providing the HCT116 line and its isogenic p53 knockout variant HCT116 p53−/−. We also thank Adele Petricca for her helpful assistance in typing the manuscript.

References

1. Morin GB (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59: 521–529
2. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S and Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. Science 279: 349–352
3. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC and Yu J (1995) The RNA component of human telomerase. Science 269: 1236–1241
4. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB and Cech TR (1997) Telomerase catalytic subunit homologs from fission yeast and human. Science 277: 955–959
5. Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, Arruda I and Robinson MO (1997) A mammalian telomerase-associated protein. Science 275: 973–977
6. Vaziri H and Benchimol S (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr. Biol. 8: 279–282
7. Fu W, Begley JG, Killen MW and Mattson MP (1999) Anti-apoptotic role of telomerase in pheochromocytoma cells. J. Biol. Chem. 274: 7264–7271
8. Karlseder J, Broccoli D, Dai Y, Hardy S and de Lange T (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science 283: 1321–1325
9. Zhang X, Mar V, Zhou W, Harrington L and Robinson MO (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev. 13: 2388–2399
10. Fu W, Killen M, Culmsee C, Dhar S, Pandita TK and Mattson MP (2000) The catalytic subunit of telomerase is expressed in developing brain neurons and serves a cell survival-promoting function. J. Mol. Neurosci. 14: 3–15
11. Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JH, Meyerson M and Weinberg RA (1999) Inhibition of telomerase limits the growth of human cancer cells. Nat. Med. 5: 1164–1170

12. Saretzki G, Ludwig A, von Zglinicki T and Runnebaum IB (2001) Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. Cancer Gene Ther. 8: 827–834
13. Kraemer K, Fuessel S, Schmidt U, Kotzsch M, Schwenzer B, Wirth MP and Meye A (2003) Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. Clin. Cancer Res. 9: 3794–3800
14. Blackburn EH (2000) Telomere states and cell fates. Nature 408: 53–56
15. Kondo Y, Kondo S, Tanaka Y, Haqqi T, Barna BP and Cowell JK (1998) Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis. Oncogene 16: 2243–2248
16. Ludwig A, Saretzki G, Holm PS, Tiemann F, Lorenz M, Emrich T, Harley CB and von Zglinicki T (2001) Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. Cancer Res. 61: 3053–3061
17. Harley CB and Sherwood SW (1997) Telomerase, checkpoints and cancer. Cancer Surv. 29: 263–284
18. Lu C, Fu W and Mattson MP (2001) Telomerase protects developing neurons against DNA damage-induced cell death. Brain Res. Dev. Brain Res. 131: 167–171
19. Ren JG, Xia HL, Tian YM, Just T, Cai GP and Dai YR (2001) Expression of telomerase inhibits hydroxyl radical-induced apoptosis in normal telomerase negative human lung fibroblasts. FEBS Lett. 488: 133–138
20. Boise LH, Gottschalk AR, Quintans J and Thompson CB (1995) Bcl-2 and Bcl-2-related proteins in apoptosis regulation. Curr. Top Microbiol. Immunol. 200: 107–121
21. Korsmeyer SJ (1995) Regulators of cell death. Trends Genet. 11: 101–105
22. Carson DA and Ribeiro JM (1993) Apoptosis and disease. Lancet 341: 1251–1254
23. Mandal M and Kumar R (1996) Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth Differ. 7: 311–3118
24. Mandal M, Maggirwar SB, Sharma N, Kaufmann SH, Sun SC and Kumar R (1996) Bcl-2 prevents CD95 (Fas/APO-1)-induced degradation of lamin B and poly(ADP-ribose) polymerase and restores the NF-kappaB signaling pathway. J. Biol. Chem. 271: 30354–30359
25. Mandal M and Kumar R (1997) Bcl-2 modulates telomerase activity. J. Biol. Chem. 272: 14183–14187
26. Catalano A, Romano M, Robuffo I, Strizzi L and Procopio A (2001) Methionine aminopeptidase-2 regulates human mesothelioma cell survival: role of Bcl-2 expression and telomerase activity. Am. J. Pathol. 159: 721–731
27. Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM, Croce CM, Alnemri ES and Huang Z (2000) Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc. Natl. Acad. Sci. USA 97: 7124–7129
28. Zangemeister-Wittke U, Leech SH, Olie RA, Simoes-Wust AP, Gautschi O, Luedke GH, Natt F, Haner R, Martin P, Hall J, Nalin CM and Stahel RA (2000) A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. Clin. Cancer Res. 6: 2547–2555
29. Gautschi O, Tschopp S, Olie RA, Leech SH, Simoes-Wust AP, Ziegler A, Baumann B, Odermatt B, Hall J, Stahel RA and Zangemeister-Wittke U (2001) Activity of a novel bcl-2/bcl-xL-bispecific antisense oligonucleotide against tumors of diverse histologic origins. J. Natl. Cancer Inst. 93: 463–471
30. Pei XY, Dai Y and Grant S (2003) The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. Leukemia 17: 2036–2045
31. Liu K, Hodes RJ and Weng Np (2001) Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. J. Immunol. 166: 4826–4830
32. Seimiya H, Sawada H, Muramatsu Y, Shimizu M, Ohko K, Yamane K and Tsuruo T (2000) Involvement of 14-3-3 proteins in nuclear localization of telomerase. EMBO J. 19: 2652–2661
33. Del Bufalo D, Biroccio A, Leonetti C and Zupi G (1997) Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line. FASEB J. 11: 947–953
34. Xu D, Wang Q, Gruber A, Bjorkholm M, Chen Z, Zaid A, Selivanova G, Peterson C, Wiman KG and Pisa P (2000) Downregulation of telomerase reverse transcriptase mRNA expression by wild-type p53 in human tumor cells. Oncogene 19: 5123–5133
35. Trisciuoglio D, Iervolino A, Candiloro A, Fibbi G, Fanciulli M, Zangemeister-Wittke U, Zupi G and Del Bufalo D (2004) bcl-2 induction of urokinase plasminogen activator receptor expression in human cancer cells through Sp1 activation: involvement of ERK1/ERK2 activity. J. Biol. Chem. 279: 6737–6745
36. Ricca A, Biroccio A, Del Bufalo D, Mackay AR, Santoni A and Cippitelli M (2000) bcl-2 over-expression enhances NF-kappaB activity and induces mmp-9 transcription in human MCF7(ADR) breast-cancer cells. Int. J. Cancer 86: 188–196
37. Minamino T and Kourembanas S (2001) Mechanisms of telomerase induction during vascular smooth muscle cell proliferation. Circ. Res. 89: 237–243
38. Haendeler J, Hoffmann J, Brandes RP, Zeiher AM and Dimmeler S (2003) Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol. Cell. Biol. 23: 4598–4610
39. Haendeler J, Hoffmann J, Diehl JF, Vasa M, Spyridopoulos I, Zeiher AM and Dimmeler S (2004) Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. Circ. Res. 94: 768–775
40. Hemann MT, Rudolph KL, Strong MA, DePinho RA, Chin L and Greider CW (2001) Telomere dysfunction triggers developmentally regulated germ cell apoptosis. Mol. Biol. Cell. 12: 2023–2030
41. Mattson MP and Klapper W (2001) Emerging roles for telomerase in neuronal development and apoptosis. J. Neurosi. Res. 63: 1–9
42. Oh H, Taffet GE, Youker KA, Entman ML, Overbeek PA, Michael LH and Schneider MD (2001) Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc. Natl. Acad. Sci. USA. 98: 10308–10313
43. Wong KK, Chang S, Weiler SR, Ganesan S, Chaudhuri J, Zhu C, Artandi SE, Rudolph KL, Gottlieb GJ, Chin L, Alt FW and DePinho RA (2000) Telomere dysfunction impairs DNA repair and enhances sensitivity to ionizing radiation. Nat. Genet. 26: 85–88
44. Zhu H, Fu W and Mattson MP (2000) The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis. J. Neurochem. 75: 117–124
45. Sharma GG, Gupta A, Wang H, Scherthan H, Dhar S, Gandhi V, Iliakis G, Shay JW, Young CS and Pandita TK (2003) hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene 22: 131–146
46. Smith LL, Coller HA and Roberts JM (2003) Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat. Cell. Biol. 5: 474–479
47. Biroccio A, Amodei S, Benassi B, Scarsella M, Cianciulli A, Mottolese M, Del Bufalo D, Leonetti C and Zupi G (2002) Reconstitution of hTERT restores tumorigenicity in melanoma-derived c-Myc low-expressing clones. Oncogene 21: 3011–3019
48. Counter CM, Hahn WC, Wei W, Caddle SD, Beijerbergen RL, Lansdorp PM, Sedivy JM and Weinberg RA (1998) Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc. Natl. Acad. Sci. USA 95: 14723–14728
49. Kosciolek BA, Kalantidis K, Tabler M and Rowley PT (2003) Inhibition of telomerase activity in human cancer cells by RNA interference. Mol. Cancer Ther. 2: 209–216
50. Biroccio A, Benassi B, Amodei S, Gabellini C, Del Bufalo D and Zupi G (2001) c-Myc down-regulation increases susceptibility to cisplatin through reactive oxygen species-mediated apoptosis in M14 human melanoma cells. Mol. Pharmacol. 60: 174–182
51. Biroccio A, Benassi B, Filomeni G, Amodei S, Marchini S, Chiorino G, Rotilio G, Zupi G and Ciriolo MR (2002) Glutathione influences c-Myc-induced apoptosis in M14 human melanoma cells. J. Biol. Chem. 27: 43763–43770
52. Biroccio A, Benassi B, Fiorentino F and Zupi G (2004) Glutathione depletion induced by c-Myc downregulation triggers apoptosis on treatment with alkylating agents. Neoplasia 6: 195–206