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# Inhibition of Telomerase Increases Resistance of Melanoma Cells to Temozolomide, but Not to Temozolomide Combined with Poly (ADP-Ribose) Polymerase Inhibitor

LUCIO TENTORI, ILARIA PORTARENA, MARCELLA BARBARINO, ALESSANDRA BALDUZZI, LAURETTA LEVATI, MATTEO VERGATI, ANNAMARIA BIROCCIO, BARRY GOLD, MARIA LUISA LOMBARDI, and GRAZIA GRAZIANI

*Department of Neuroscience, University of Rome "Tor Vergata", Rome, Italy (L.T., I.P., M.B., A.B., M.V., G.G.); "Istituto Dermopatico dell'Immacolata", Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy (L.L.); Experimental Oncology-Immunology, National Cancer Institute "G. Pascale", Naples, Italy (M.B., M.L.L.); Eppley Institute for Research in Cancer and Allied Diseases and Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska (B.G.); and Experimental Chemotherapy Laboratory, "Regina Elena" Cancer Institute, Rome, Italy (A.B.)*

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## ABSTRACT

In the present study, we have investigated the influence of telomerase inhibition in chemosensitivity of melanoma cells to temozolomide (TMZ), a methylating agent with promising antitumor activity against metastatic melanoma. In fact, telomerase, a ribonucleoprotein enzyme expressed in the majority of tumors, is presently considered an attractive target for anticancer therapy, with the double aim of reducing tumor growth and increasing chemosensitivity of cancer cells. Susceptibility to TMZ and to other antitumor agents used for treatment of metastatic melanoma was initially assessed in melanoma lines with different basal levels of telomerase activity. Thereafter, chemosensitivity was investigated after inhibition of telomerase by means of stable transfection of a catalytically inactive, dominant-negative mutant of hTERT (DN-hTERT). This study shows for the first time that: a) susceptibility to TMZ of melanoma lines derived from the same patient did not depend on basal telomerase activity; b) inhibition of telomerase by DN-hTERT resulted in reduced growth rate and increased resistance to TMZ and to the chloroethylating agent carmustine, increased sensitivity to cisplatin, and no change in response to tamoxifen or to a selective *N*3-adenine methylating agent; c) inhibition of poly(ADP-ribose) polymerase (PARP), an enzyme involved in the repair of *N*-methylpurines, restored sensitivity of DN-hTERT clones to TMZ. These results indicate that a careful selection of the antitumor agent has to be made when antitelomerase therapy is combined with chemotherapy. Moreover, the data presented here suggest that TMZ + PARP inhibitor combination is active against telomerase-suppressed and slowly growing tumors.

The incidence of cutaneous melanoma is rapidly increasing throughout the world. Although at early stages melanoma can be cured by surgical resection, the prognosis of metastatic melanoma is poor and no treatment currently available substantially affects the course of the disease. Few chemotherapeutic agents have shown activity in patients with metastatic melanoma; among these, the methylating agent dacarbazine (DTIC) is still considered the reference drug even though the response rate is only about 20% (Balch et al., 1997).

Recently, temozolomide (TMZ), a novel oral methylating agent in a phase III study enrolling patients affected by metastatic melanoma without central nervous system involvement, has shown efficacy equal to that of DTIC (Middleton et al., 2000). Because of its ability to cross the blood-brain barrier, TMZ has been reported to reduce the incidence of central nervous system relapses (Abrey and Christodoulou, 2001) and to have activity against brain metastasis (Bleehen et al., 1995; Biasco et al., 2001). In contrast to DTIC, TMZ does not require metabolic activation and is devoid of severe adverse effects (Newlands et al., 1997).

Both agents are prodrugs of the active methylating species

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**ABBREVIATIONS:** DTIC, dacarbazine; TMZ, temozolomide; AGT, $O^6$-alkylguanine DNA alkyltransferase; MR, mismatch repair; PARP, poly(ADP-ribose) polymerase; BER, base excision repair system; MPG, 3-methyladenine-DNA glycosylase; hTERT, human telomerase reverse transcriptase; DN-hTERT, dominant negative mutant of human telomerase reverse transcriptase; Lex, $MeOSO_2(CH_2)_2$-Lexitropsin; AB, 3-aminobenzamide; BCNU, 1, 3-bis(2-chloroethyl)-1-nitrosourea; TAM, tamoxifen; CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium; TRAP, telomeric repeats amplification protocol; PCR. polymerase chain reaction; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; AEBSF, 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride; kb, kilobase(s).

5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, which interacts with DNA. Even though 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide produces a wide spectrum of methyl adducts, mostly represented by *N*-methylpurines, its cytotoxic activity has been mainly attributed to methylation of the $O^6$ position of guanine despite the fact that this lesion accounts for only a small percentage of total DNA adducts (Newlands et al., 1997).

Although the results of clinical trials with TMZ are promising, the efficacy of $O^6$-methylating agents is strongly influenced by the functional status of DNA repair systems, such as $O^6$-alkylguanine DNA alkyltransferase (AGT), which removes the methyl adduct from the $O^6$-position of guanine (Pegg, 2000) and mismatch repair (MR). The latter is required for the induction of DNA strand breaks, growth arrest and/or apoptosis (D'Atri et al., 1998; Hirose et al., 2001).

In AGT-proficient cells, depletion of AGT activity by $O^6$-benzylguanine, a specific inhibitor of the enzyme, restores sensitivity to $O^6$-alkylating agents (Dolan and Pegg, 1997). However, in the presence of functional defects of MR pathway, even AGT-deficient cells are tolerant to cytotoxicity induced by $O^6$-methylguanine (D'Atri et al., 1998). In MR-deficient tumors, resistance can be abrogated by inhibitors of poly(ADP-ribose) polymerase (PARP), a component of base excision repair system (BER) (Wedge et al., 1996; Tentori et al., 1997, 1999, 2002a; Liu et al., 1999). In the presence of PARP inhibitor, cytotoxicity is caused by interruption of the repair process of *N*-methylpurines generated by TMZ after the initial removal of the methylated bases by 3-methyladenine-DNA glycosylase (MPG).

Recently, it has been demonstrated that resistance of tumor cells to chemotherapy can be also attributed to elevated telomerase expression (Faraoni et al., 2000; Mergny et al., 2002). Telomerase is a ribonucleoprotein DNA polymerase that adds telomeric repeats at the end of chromosomes, compensating for the gradual loss of telomeric sequences that occurs during cell division (Morin, 1989). When a critical telomere length is reached, cells undergo senescence and stop proliferating (Hahn et al., 1999). Telomerase is composed of a catalytic subunit, hTERT, and a template RNA component and is overexpressed in a large number of tumors, whereas it is not expressed in most somatic cells. Because inhibition of telomerase activity results in suppression of tumor cell growth and increased apoptosis, telomerase is presently considered an attractive target for the development of novel anticancer agents (Mergny et al., 2002). Although a number of studies have evaluated the influence of telomerase suppression on tumor cell growth, few studies have assessed the possible interactions between telomerase inhibitors and chemotherapy.

In the present study, we investigated the susceptibility to TMZ ± PARP inhibitor of melanoma cell lines, endowed with different levels of telomerase activity. Moreover, susceptibility to drug treatment was assessed upon telomerase inhibition by means of stable transfection of a catalytically inactive, dominant-negative mutant of hTERT (DN-hTERT). Chemosensitivity to other antitumor agents used for the treatment of metastatic melanoma was also analyzed.

The results indicate that in melanoma cell lines derived from the same patient and characterized by different levels of telomerase and AGT activity, susceptibility to TMZ depended not on basal telomerase but on AGT levels. Inhibition of telomerase in melanoma cell lines with comparable AGT activity resulted in reduced growth rate and increased resistance to TMZ and carmustine, but augmented sensitivity to cisplatin. Interestingly, inhibition of PARP restored sensitivity of DN-hTERT clones to TMZ.

## Materials and Methods

**Cell Lines.** PES43, PES47, CIMA62, and CIMA73 melanoma cell lines were obtained as described previously (Pirozzi et al., 2000). In brief, tumor tissues, suspended in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 80 $\mu$g/ml gentamicin, and 2 mM L-glutamine, were minced with scalpels to a fine suspension, centrifuged at 180*g* for 5 min at room temperature, resuspended in fresh complete medium, and cultured at 37°C in a 5% $CO_2$ humidified atmosphere. The human melanoma cell lines PES43, PES47, CIMA62, and CIMA73 were obtained from distinct metastatic lesions derived from the two different patients, P.E. (for PES) and C.M. (for CIMA). The human melanoma cell lines M14 and MAS51, a M14-derived clone expressing low levels of *c-myc*, were obtained as described previously (Biroccio et al., 2001).

Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (Flow Laboratories, McLean, VA), at 37°C in a 5% $CO_2$ humidified atmosphere.

**Drug Treatment and Cell Growth Evaluation.** TMZ was kindly provided by Shering Plough Research Institute (Kenilworth, NJ). $MeOSO_2(CH_2)_2$-*N*-methylpyrrole dipeptide [$MeOSO_2(CH_2)_2$-Lexitropsin (Lex)] was prepared as described previously (Zhang et al., 1993). Inhibition of PARP was obtained by treating the cells with 4 mM 3-aminobenzamide (AB; Sigma, St. Louis, MO), a concentration that has been described to completely inhibit PARP activity (Tentori et al., 1999).

Drug stock solutions were prepared by dissolving TMZ in dimethyl sulfoxide, carmustine, or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, Bristol-Myers Squibb, Princeton, NJ), Lex, tamoxifen (TAM; Sigma) in ethanol, *cis*-diamminedichloroplatinum(II) [cisplatin (CDDP); Teva, Pharm ltd., Milan, Italy) in 0.9% NaCl solution, and AB in culture medium. The final concentration of dimethyl sulfoxide or ethanol in drug-treated cultures was always less than 0.1% (v/v) and did not contribute to toxicity (data not shown). The concentrations tested were as follows: TMZ, 3.5 to 1000 $\mu$M; Lex, 1.5 to 50 $\mu$M; BCNU, 0.2 to 20 $\mu$M; TAM, 0.5 to 10 $\mu$M; and CDDP, 0.2 to 6 $\mu$M. For each drug, concentrations always included the peak plasma concentration that can be reached in patients.

Cells were cultured in flasks (BD Falcon; BD Discovery Labware, Oxnard, CA) ($2 \times 10^5$ cells/flask). Adherent cells were treated with graded concentrations of the drugs under study or with drug solvent only. Cells were then incubated at 37°C for 3 days and growth was evaluated by counting cells in quadruplicate. Cell viability was determined by trypan blue dye exclusion.

Cell proliferation was also evaluated 5 days after drug treatment using a Promega kit, according to the manufacturer's instructions (CellTiter 96 Aqueous one solution cell proliferation assay; Promega, Madison, WI). The assay uses the novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS).

Long-term cell survival was determined by means of colony-forming assay. Drug treated cells ($2 \times 10^2$) were seeded into six-well plates (BD Falcon) to allow colony formation. After 10 days, untreated and drug-treated colonies were fixed and stained with rhodamine B basic violet 10 (ICN Biomedicals Inc., Aurora, OH). Survival was calculated as percentage of untreated control. Cell line chemosensitivity was evaluated in terms of $IC_{50}$ (i.e., the micromolar concentration of the drug capable of inhibiting colony-forming ability

by 50%). The $IC_{50}$ was calculated on the regression line in which the number of colonies was plotted versus the drug concentration.

**Generation of Amphotropic Retroviruses.** The pBABE-puro and pBABE-puro-DN-hTERT were kindly provided by Dr. Robert Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). DN-hTERT is a catalytically inactive, dominant-negative form of hTERT obtained by substituting the aspartic acid and alanine residues at position 710 and 711 of hTERT with valine and isoleucine residues, respectively (Hahn et al., 1999). Amphotropic retroviruses were obtained by transfection of the RetroPack PT67 cell line (BD Clontech, Palo Alto, CA) using CalPhos Mammalian transfection Kit (BD Clontech), according to the manufacturer's instructions. The virus-containing supernatants were collected, filtered through a 0.45-$\mu$m cellulose acetate filter, and used to infect melanoma cells in the presence of 8 $\mu$g/ml polybrene. Cells were selected in puromycin (2.5 $\mu$g/ml) and clones isolated by ring cloning.

**Telomerase Assay.** The telomeric repeats amplification protocol (TRAP) assay, based on PCR amplification of telomerase extension products, was performed as described previously (Piatyszek et al., 1995). Extracts were prepared by lysing the cells in ice-cold extraction buffer [0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.25 mM sodium deoxycholate, 150 mM NaCl, 10% glycerol, 5 mM $\beta$-mercaptoethanol, and 0.1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF)]. Four microliters of cell extracts, corresponding to 150 to 4000 cells or to 100 to 400 ng, were used for TRAP assay.

The telomerase reaction was carried out in 40 $\mu$l of the reaction mixture, consisting of 20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.05% Tween 20, 0.1 $\mu$g of TS primer (5′-AATCCGTCGAGCAGAGTT–3′), 0.1 $\mu$M T4 gene 32 protein, and 50 $\mu$M concentrations of each deoxynucleotide triphosphate. Samples were incubated at room temperature for 15 min to allow telomerase to extend TS primer. The reaction was stopped in ice and 2 units of *Taq*DNA polymerase, 0.16 $\mu$l of $\alpha$[$^{32}$P]dCTP (3000 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) and 0.1 $\mu$g of CX oligonucleotide (5′-CCCTTACCCTTACCCTTACCCTAA-3′) were added to each single PCR tube. Amplification of the telomeric products was performed by PCR (94°C, 30 s; 50°C, 30 s; 72°C, 1 min; 31 cycles). After TRAP assay, 40 $\mu$l of the PCR reaction was separated on a 10% nondenaturing polyacrylamide gel. Subsequently, gels were fixed and exposed to X-ray films (Eastman Kodak, Rochester, NY) at −80°C. The signal of the telomeric ladder was quantified by bidimensional densitometry using a Bio-Rad scanning apparatus (Imaging densitometer, GS-670; Molecular Analyst software) and each value was corrected for the background (i.e., lane relative to lysis buffer).

Assay of alkaline phosphatase activity as an internal control for the quality of the cell extract was performed using a commercially available kit (Sigma) as described previously (Piatyszek et al., 1995).

**RT-PCR.** Analysis of the expression of the retrovirally encoded hTERT was performed by RT-PCR. Total RNA was treated with RNase-free DNase and cDNA was synthesized by incubating 1.5 $\mu$g RNA with 0.5 U of avian myeloblastosis virus RT and 0.2 $\mu$g of oligo(dT) primer at 42°C for 1 h, using the cDNA Cycle Kit from Invitrogen. Aliquots (5 $\mu$l) of the reverse-transcribed cDNA were subjected to 30 cycles of PCR in 50 $\mu$l of 1× buffer (10 mM Tri-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl) containing 1 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 $\mu$Ci of $\alpha$[$^{32}$P]dCTP, 2.5 U of *Taq*DNA polymerase (Roche, Milan, Italy), and 0.2 mM of specific primers. Each cycle consisted of denaturation at 94°C for 45 s, annealing at 60°C (hTERT) or 53°C [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] for 45 s, and extension at 72°C for 90 s. The primer pairs used for retrovirally encoded hTERT were 5′-CTGCTACTCCATCCTGAAAGC-3′ and 5′-TTGCATACTTCTGCCTGCTGG-3′ and amplified a 345-base pair fragment. The primers used for GAPDH amplification (5′-TGGTATCGTGGAAGGACTCATGAC-3′ and 5′ATGCCAGTGAGCTTCCCGTTCAGC-3′) amplified a 190-base pair product. PCR reaction (20 $\mu$l) were electrophoresed through a 2% agarose gel containing ethidium bromide.

**Telomere Analysis.** High molecular weight DNA was obtained by phenol/chloroform extraction. *Hin*fI-digested DNA (5 $\mu$g) was separated by 0.7% agarose gel electrophoresis and transferred to Hybond N plus membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using 0.4 N NaOH as transfer buffer. Staining of the gels with ethidium bromide confirmed digestion and transfer of DNA. Membranes were hybridized with a telomeric biotinylated oligonucleotide probe $(TTAGGG)_5$ (Invitrogen) at 45°C, using the North2South chemiluminescent hybridization and detection kit (Pierce Biotechnology, Rockford, IL). Filters were exposed to BioMax MR autoradiographic films (Kodak). After densitometric analysis, the mean TRF length for each sample was calculated using the formula: $\Sigma\ (OD_i \times L_i)/\Sigma(OD_i)$, where $OD_i$ is the densitometer output from grid box i, and $L_i$ is the size of DNA at position i relative to markers.

**Western Blot Analysis.** Cell lysates were prepared as described previously (Tentori et al., 1999). Proteins (80 $\mu$g/sample) were electrophoresed in 8% SDS-polyacrylamide mini-gels. Afterward, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Equal protein loading was visualized by Ponceau S staining. Filters were blocked with blocking buffer (Roche) and incubated with monoclonal antibodies directed against human MLH1 (Ab-1), MSH2 (Ab-1) (BD Clontech) or actin (Sigma). Immunecomplexes were visualized using a chemiluminescence kit (Amersham Biosciences), according to the manufacturer's instructions. Filters were exposed to X-OMAT AR autoradiographic films (Kodak) for 10 to 45 s, depending on the intensity of the signal.

**Analysis of AGT Activity.** Cells were washed twice with phosphate-buffered saline, centrifuged at 130*g*, and stored as pellets ($2 \times 10^6$) at −80°C until used. Cells were suspended in 0.5 ml of a buffer containing 0.5% CHAPS, 50 mM Tris-HCl pH 8, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 200 $\mu$g/ml leupeptin, 5 $\mu$g/ml aprotinin, 400 $\mu$g/ml soybean trypsin inhibitor, and 1 mM AEBSF and incubated at 4°C for 30 min. Cell lysates were then centrifuged at 15,000 rpm at 4°C for 10 min, and supernatants were immediately used for the assay. Various amounts of cell extracts were incubated with 10 $\mu$g of calf thymus DNA methylated by *N*-[$^3$H]methyl-*N*-nitrosourea (19 Ci/mmol; Amersham Biosciences), and AGT activity was determined by measuring the transfer of [$^3$H]methyl groups from methylated DNA to the AGT protein. DNA was then hydrolyzed by heating samples at 75°C for 45 min in the presence of 1 N perchloric acid and proteins precipitated with 1 milligram of bovine serum albumin as carrier. Pellets were then washed with 1 N perchloric acid and suspended in 0.01 N NaOH. Radioactivity was counted in a scintillation counter after the addition of scintillation liquid (Ultima Gold; PerkinElmer Life Sciences, Zaventem, Belgium). Protein concentration of cell extracts was evaluated according to the method of Bradford (1976) using the Bio-Rad dye solution and bovine serum albumin as standard. AGT activity was expressed as femtomoles of methyl groups per milligram of protein in the cell extract.

**Measurement of MPG Activity.** MPG activity was assayed as described previously (Tentori et al., 2002b). Tumor cells ($10^7$) were sonicated at 4°C in 0.5 ml of buffer I (50 mM Tris-HCl, 3 mM dithiothreitol, and 2 mM EDTA, pH 8.3), with freshly added 1 mM AEBSF. After removal of cell debris by centrifugation, supernatants were immediately tested for MPG activity. Various amounts of cell extracts were incubated with 15 $\mu$g (15,000 cpm) of freshly dissolved calf thymus DNA methylated by *N*-[$^3$H]methyl-*N*-nitrosourea (19 Ci/mmol; Amersham Biosciecnes) in a total volume of 100 $\mu$l of buffer II (20 mM Tris-HCl, 1 mM dithiothreitol, 60 mM NaCl, and 1 mM EDTA, pH 8). After 1 h at 37°C, the reaction was stopped on ice by the addition of 30 $\mu$l of 2 M NaCl containing 0.5 mg/ml calf thymus DNA and 1 mg/ml bovine serum albumin. DNA was ethanol-precipitated and samples were centrifuged at 10,000*g* for 15 min. Three hundred microliters of the supernatants was transferred to a scin-

tillation tube and counted. MPG activity was determined for protein and time-limiting conditions and expressed as femtomoles of methylpurines released per milligram of protein per hour.

## Results

**Analysis of Chemosensitivity Profile in Melanoma Cell Lines with Different Basal Levels of Telomerase and DNA Repair Enzyme Activity.** Melanoma cell lines were initially tested for basal telomerase expression. It should be noted that PES43 and PES47 melanoma cell lines were derived from two different metastases of the same patient (Pirozzi et al., 2000). Analogously, CIMA62 and CIMA73 were obtained from distinct localizations of metastatic melanoma in another patient (Pirozzi et al., 2000). The melanoma MAS51 cell line was instead obtained by transfection of M14 cells with an expression vector carrying the *c-myc* gene in antisense orientation (Biroccio et al., 2001). Analysis of telomerase activity by TRAP assay, using graded number of cells, indicated that PES43 and CIMA62 expressed lower levels of telomerase activity compared with PES47 and CIMA73, respectively (Fig. 1A). Moreover, MAS51 cells showed barely detectable levels of telomerase activity with respect to the parental M14 cell line (Fig. 1A), in accordance with a previous study (Biroccio et al., 2002).

Despite the differences in telomerase activity, PES43 and PES47 or CIMA62 and CIMA73 were characterized by similar growth rates. In contrast, MAS51 cells showed a reduction in the proliferation rate with respect to M14 line (Biroccio et al., 2001).

Analysis of telomere length by Southern blot hybridization indicated that PES43 cells possessed shorter telomeres than PES47 cells (average telomere length: PES43, 3.7 kb; PES47, 5 kb), whereas CIMA 62 and CIMA 73 showed comparable telomere length (average telomere length: CIMA62, 2.4 kb; CIMA73, 2.3 kb) (Fig. 1B). Moreover, it has been previously demonstrated that MAS51 cells (average telomere length, 4 kb) have shorter telomeres than M14 cells (average telomere length, 8 kb) (Biroccio et al., 2002). Telomere size detected in PES- or M14-derived cell lines was within the range reported for other malignant melanoma cells (4–14 Kb) (A. Biroccio, personal communication; Parris et al., 1999; Folini et al., 2000). CIMA cells possessed, instead, very short telomeres.

Melanoma lines were also characterized for the levels of AGT and MPG activity. The results, illustrated in Fig. 2A, indicate that PES43 and PES47 possessed similar levels of AGT activity. On the contrary, MAS51 and CIMA73 showed significantly higher levels of AGT compared with M14 and CIMA62 lines, respectively. No significant differences in the levels of MPG activity were instead observed among cell lines of the same origin.

Melanoma cell lines were exposed to graded concentrations of the alkylating agents TMZ and BCNU. The latter was tested, because BCNU efficacy, as in the case of TMZ, inversely correlates with AGT levels (Pegg, 2000). BCNU forms a chloroethyl adduct at the $O^6$ position of guanine, which, if not repaired by AGT, generates a covalent interstrand DNA cross-link between the modified guanine and the opposite cytosine. Susceptibility of melanoma cells to the *N*3-adenine selective methylating agent Lex was also investigated. This agent inhibits tumor cell growth even in the case of poor responsiveness to TMZ and susceptibility to Lex depends mainly on MPG levels (Encell et al., 1996; Tentori et al., 2000, 2001a). TMZ or Lex were tested in association with the PARP inhibitor AB, which is known to enhance tumor growth inhibition induced by both methylating agents (Tentori et al., 2000, 2001a, 2002a).

Cell growth was evaluated by colony-forming assay and chemosensitivity was expressed in terms of $IC_{50}$ (Table 1). PES43 and PES47 cell lines, which possess similar levels of AGT activity, showed comparable chemosensitivity to TMZ and BCNU. AGT-deficient/telomerase-deficient CIMA62 line was more susceptible to TMZ and BCNU with respect to AGT-proficient/telomerase positive CIMA73. Accordingly, AGT-deficient but telomerase-positive M14 was more sensitive to TMZ and BCNU than the AGT-proficient/telomerase-deficient MAS51 line. Thus, TMZ and BCNU chemosensitivity did not seem to be dependent on basal telomerase expression; rather, it seemed to correlate with AGT levels. It must be noted that PES lines were 3-fold more resistant to TMZ than were MAS51 cells, even though they possessed

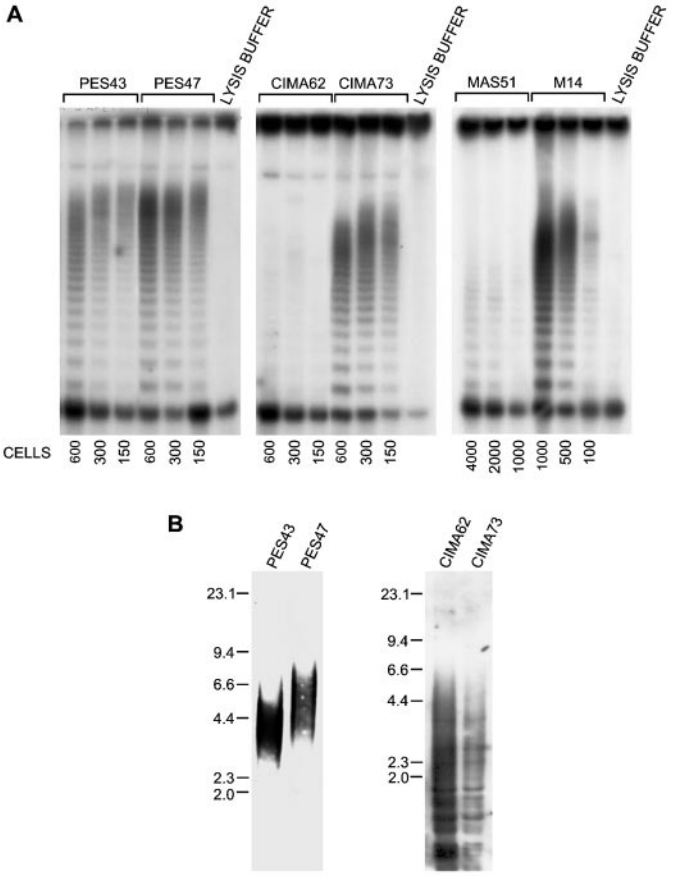


**Fig. 1.** Telomerase expression and telomere length in melanoma cell lines. A, analysis of basal telomerase activity by TRAP assay. Telomerase activity was assessed using graded number of cells in PES43 and PES47, CIMA62 and CIMA73, and M14 and MAS51 cell lines. Negative control, cell extract was replaced by an equal volume of lysis buffer. Densitometric analysis of the entire telomeric ladder was performed, and optical densities corresponding to equal number of cells were compared. The results indicate that in PES43, CIMA62, and MAS51 telomerase expression was 60, 10, and 3% of that detected in PES 47, CIMA73, and M14 cells, respectively. When densitometric analysis of large or small fragments was performed, mean optical density values in PES43 cells were 70 ± 10 and 50 ± 16%, respectively, of those detected in PES47 cells. B, measurement of telomere length by telomere restriction fragment analysis in PES43, PES47, CIMA62, and CIMA73 cells. Molecular weight markers expressed in kilobases (kb): λ DNA-Hind III (Invitrogen).

similar levels of AGT activity. Because MR deficiency can be responsible of poor responsiveness to TMZ, we have analyzed the expression of MR by Western blot analysis of MLH1 and MSH2 proteins. These proteins are the two components of MR system most frequently mutated in human malignancies and can be also defective in malignant melanoma (Lage et al., 1999; Korabiowska et al., 2000; Lo Muzio et al., 2000). The results indicate that MSH2 and MLH1 proteins were expressed in all melanoma cell lines tested, although at different levels (Fig. 2B).

PES43 and PES47, CIMA62 and CIMA73, or M14 and MAS51 cell lines showed comparable susceptibility to Lex (Table 1). This is consistent with similar MPG levels detected in each pair of cell lines (Fig. 2A). Finally, it is interesting to note that in all cases, the PARP inhibitor AB potentiated 2- to 3-fold growth inhibition induced by the methylating agents TMZ and Lex (Table 1).

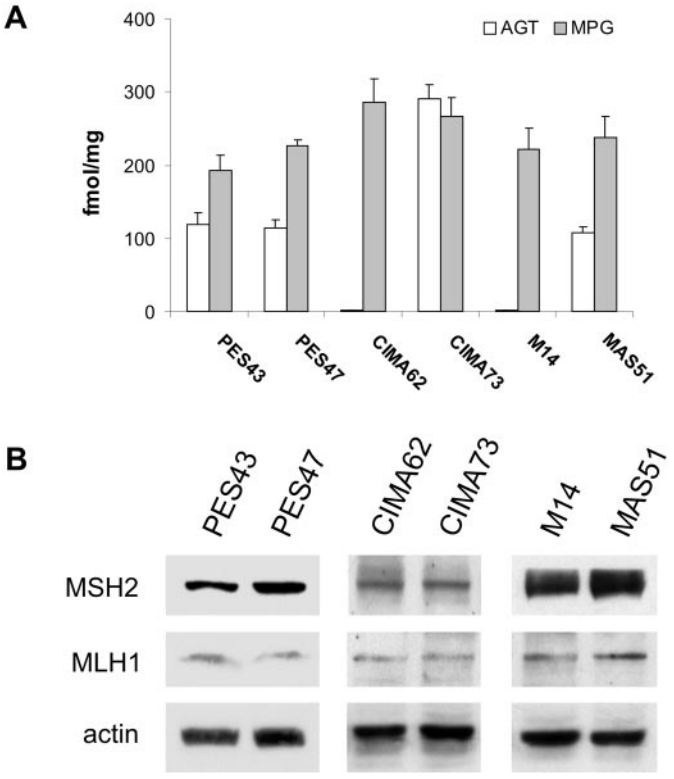


**Fig. 2.** Pattern of expression of DNA repair enzymes in melanoma cell lines. A, analysis of AGT and MPG activities. AGT activity is expressed as femtomoles of methyl groups per milligram of total proteins. Each histogram represents the mean of four independent determinations + S.E. MPG activity is expressed as femtomoles of methylpurines released per milligram of protein per hour. Values represent the mean of four determinations performed using different sample dilutions of the same cell extract. Data are representative of one of three different experiments with comparable results. B, Western blot analysis of MLH1 and MSH2 proteins. Proteins (80 $\mu$g) were size fractionated electrophoretically, transferred to a membrane, and incubated with antibodies against MSH2, MLH1, and actin. The data are representative of one of two independent experiments with similar results.

**Transfection of DN-hTERT Reduces Telomerase Activity and Growth Rate of Melanoma Cell Lines.** Inhibition of telomerase has been shown to induce a decrease in tumor cell proliferation (Hahn et al., 1999; Zhang et al., 1999). Because slow growth rate is involved in the poor responsiveness of solid tumors to chemotherapy, we have investigated the influence of telomerase inhibition in modulating proliferation and response to TMZ of melanoma cells. PES lines were chosen for this study because, unlike the other cell lines tested, they showed comparable AGT activity and susceptibility to TMZ (Table 2). Moreover, both lines expressed basal telomerase activity, although at different levels, and showed similar growth rate. PES43 and PES47 were infected with an amphotropic retrovirus encoding a catalytically inactive, dominant-negative mutant of hTERT. In fact, this mutant has been shown to reduce existing telomerase activity in a number of tumor cell lines (Hahn et al., 1999). After infection with DN-hTERT or with a control vector expressing only the puromycin resistance gene (pBABE), five drug-selected clones for each cell line and construct were analyzed for telomerase activity by the TRAP assay. A consistent reduction of telomerase activity, ranging from 50 to 95%, was observed in all DN-hTERT clones analyzed and clones with at least 70% reduction of enzymatic activity were chosen for this study. In contrast, all pBABE clones showed telomerase activity comparable with that detected in the parental cell line. Figure 3A shows the results of TRAP assay performed with DN-hTERT clones, derived from PES43 and PES47 cell lines, in which a remarkable reduction of telomerase activity was detected with respect to their pBABE control cells (85–95% in PES43 DN-hTERT clones, and 75–80% in PES47 DN-hTERT clones). Comparable levels of alkaline phosphatase activity were found in all samples (data not shown). This enzyme possesses stability similar to that of telomerase, thus serving as an internal control for the quality of the extracts (Piatyszek et al., 1995).

The expression of DN-hTERT in the clones with reduced telomerase activity was confirmed by RT-PCR, using primers that specifically allow amplification of the retrovirally encoded hTERT. The results indicate that retrovirally derived DN-hTERT transcript was present only in DN-hTERT clones, whereas GAPDH amplification was obtained in all samples (Fig. 3B).

TABLE 1

Chemosensitivity of PES-, CIMA-. and M14- derived melanoma cell lines assessed by colony-forming assay

For each cell line, AB + TMZ $IC_{50}$ was always significantly lower than TMZ $IC_{50}$ ($P < 0.001$). BCNU $IC_{50}$ of CIMA62 was significantly different from that of CIMA73; BCNU $IC_{50}$ of M14 was significantly different from that of MAS51 ($P < 0.001$). No significant differences were observed between BCNU $IC_{50}$ of PES43 and PES47 cell lines. For each cell line, AB + Lex $IC_{50}$ was always significantly lower than Lex $IC_{50}$ ($P < 0.001$).

| Cell Line | TMZ | AB + TMZ | BCNU | Lex | AB + Lex |
|---|---|---|---|---|---|
| | | | $\mu M$ | | |
| PES43 | 210 ± 6 | 68 ± 10 | 14 ± 4 | 16 ± 3 | 4.7 ± 0.7 |
| PES47 | 220 ± 24 | 87 ± 14 | 9 ± 0.6 | 14.7 ± 0.9 | 4.7 ± 0.1 |
| CIMA62 | 15 ± 2 | 3.8 ± 0.6 | 0.6 ± 0.2 | 13.9 ± 0.5 | 6 ± 0.4 |
| CIMA73 | 80 ± 4 | 36.5 ± 0.5 | 1.6 ± 0.3 | 14.3 ± 0.8 | 4.6 ± 1 |
| M14 | 24.9 ± 1 | 8.8 ± 0.1 | 0.3 ± 0.01 | 21.8 ± 0.2 | 10 ± 2 |
| MAS51 | 68.4 ± 5 | 28 ± 0.5 | 1.7 ± 0.1 | 22 ± 0.2 | 8 ± 1 |

After clonal expansion, the growth rate of control, pBABE, and DN-hTERT clones was measured by counting viable cells. Strong attenuation of telomerase activity in DN-hTERT clones was associated with a reduction of the growth rate that started to appear 2 to 3 weeks after infection. Indeed, DN-hTERT clones had significantly longer doubling times than parental or pBABE cells (Table 3), even though they never stopped proliferating. Indeed, telomere length of transfectant clones remained unchanged during the culture time (data not shown).

**Decrease of Telomerase by DN-hTERT Expression Reduces Melanoma Cell Susceptibility to TMZ but Not to TMZ + PARP Inhibitor.** Before assessing susceptibility of transfected clones to TMZ, pBABE, and DN-hTERT, clones from PES43 and PES47 lines were tested for the expression of AGT activity and MR proteins. In all clones, no significant differences in AGT activity (Table 3) or MLH1 and MSH2 expression (data not shown) were found with respect to the parental cell line.

Analysis of chemosensitivity to TMZ, used as a single agent or combined with PARP inhibitor, was evaluated by cell count and by colony-forming assay 3 and 10 days, respectively, after treatment. For each cell line the TMZ $IC_{50}$ was calculated and the results were expressed as percentage of PES43 or PES47 parental cell $IC_{50}$ (Fig. 4). All DN-hTERT clones were significantly more resistant to growth inhibition induced by TMZ with respect to parental cells or to pBABE clones: TMZ $IC_{50}$ values increased roughly 2- to 4-fold (Fig. 4). It should be noted that chemosensitivity of PES43/47 cells infected with pBABE vector was comparable with that of parental nonselected clones (data not shown).

A 3- to 4-fold increase of resistance to the methylating agent in DN-hTERT clones was also detected using MTS assay performed 5 days after drug treatment (data not shown).

Addition of the PARP inhibitor AB markedly potentiated susceptibility to TMZ of all melanoma clones tested (Fig. 5). Remarkably, the enhancement of growth inhibition was more pronounced in DN-hTERT clones than in pBABE or parental cells (Fig. 5). An example of increased resistance to TMZ as a consequence of DN-hTERT expression and the potentiating effect of PARP inhibitor is given in Fig. 6A, which illustrates the survival curves of representative PES47 pBABE and DN clones, evaluated by colony-forming assay.

In contrast to the results obtained with TMZ, susceptibility of PES47 or PES43 clones to the *N*3-adenine–selective methylating agent Lex was not affected by DN-hTERT expression. However, PARP inhibitor significantly potentiated growth inhibition induced by Lex (Fig. 6B).

Reduced responsiveness to TMZ was also observed in AGT-deficient M14 cells transduced with the DN-hTERT amphotropic virus (Fig. 7). Also, in M14 model, DN-hTERT markedly inhibited telomerase activity. Moreover, AGT activity of DN-hTERT clones remained undetectable as observed in their telomerase-positive counterparts.

Chemosensitivity of pBABE and DN-hTERT PES43 or PES47 clones to BCNU, CDDP, and TAM was then assayed, and $IC_{50s}$ were compared with those evaluated in parental cell lines. CDDP generates platinum DNA adducts at the *N*7 position of guanine, provoking mostly intrastrand cross-links. TAM is an estrogen receptor blocker whose antitumor

TABLE 2
Telomerase, AGT activity, and sensitivity to TMZ in melanoma cell lines

| Cell Lines | Telomerase Activity | AGT Activity | Sensitivity to TMZ |
|---|---|---|---|
| PES43 | –/+ | + | PES43= PES47 |
| PES47 | + | + | |
| CIMA62 | – | – | CIMA62>CIMA73 |
| CIMA73 | + | + | |
| M14 | + | – | M14> MAS51 |
| MAS51 | – | + | |

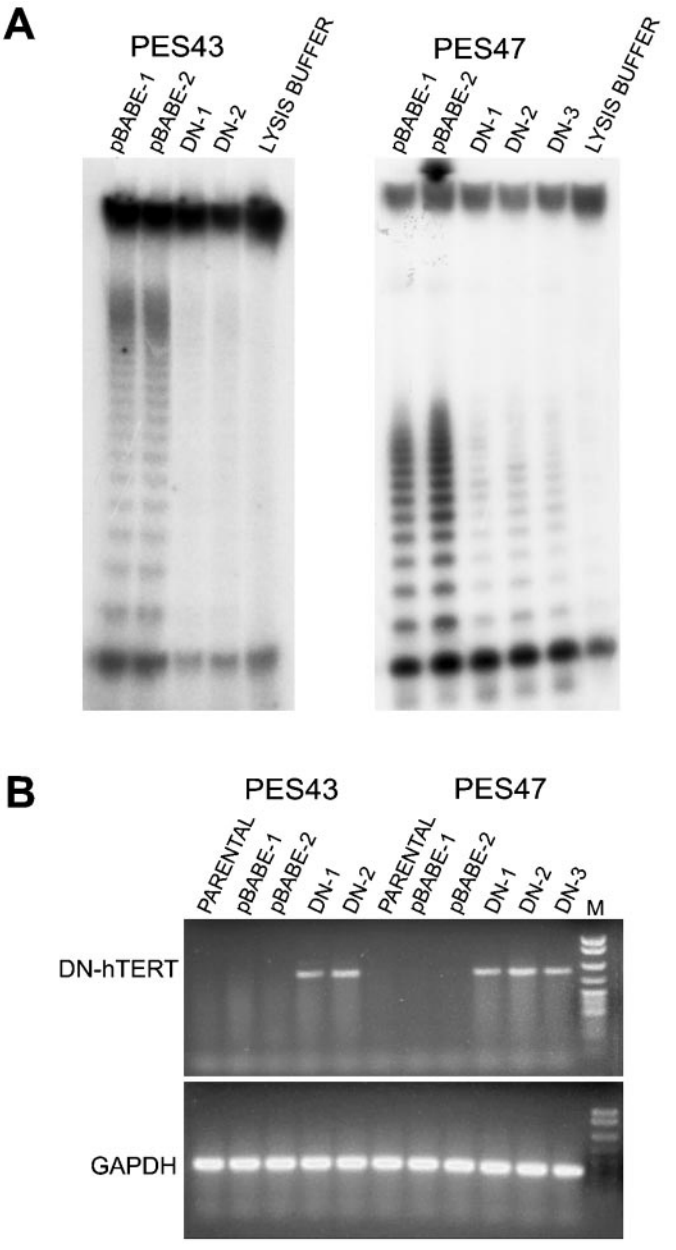


**Fig. 3.** Influence of DN-hTERT expression on telomerase activity of PES43 and PES47 melanoma cells. A, analysis of telomerase activity in clones derived from melanoma cell lines infected with control vector (pBABE) or with DN-hTERT virus (DN). Telomerase activity was tested by TRAP assay using 100 ng of proteins. Negative control, cell extract was replaced by an equal volume of lysis buffer. B, RT-PCR analysis of retrovirally derived DN-hTERT transcript. RNA samples were subjected to RT-PCR analysis, using primers specific for the retrovirally encoded DN-hTERT or for GAPDH. The experiment was repeated once with comparable results. M, pBR322 plasmid digested with *Msp*I (Invitrogen).

activity against melanoma cells is not exclusively mediated by its antiestrogen properties but probably involves modulation of the insulin-like growth factor-1 receptor signaling pathway. The results indicate that DN-hTERT clones were more resistant to growth inhibition induced by BCNU (Fig. 8A). On the contrary, DN-hTERT clones were more susceptible to CDDP with respect to parental and pBABE control cells (Fig. 8B). In this regard, it should be noted also that M14-derived DN-hTERT clones were 2- to 3-fold more sensitive to CDDP with respect to parental or pBABE clones (data not shown). Finally, DN-hTERT expression did not significantly affect sensitivity to TAM (Fig. 8C).

TABLE 3
Doubling time and AGT activity of PES43 and PES47 clones expressing control (pBABE) or DN-hTERT virus
Statistical analysis was performed according to Student's *t* test.

| Cell Lines | Doubling Time | P | AGT | P |
|---|---|---|---|---|
| | *h* | | *fmol/mg* | |
| PES43 | 42 ± 0.5 | | 119 ± 16 | |
| PES43-pBABE-1 | 41 ± 1 | N.S. | 116 ± 3 | N.S. |
| PES43-pBABE-2 | 43 ± 3 | N.S. | 104 ± 0.1 | N.S. |
| PES43-DN-1 | 70 ± 2 | = 0.0001 | 100 ± 13 | N.S. |
| PES43-DN-2 | 72 ± 2 | <0.0001 | 90 ± 15 | N.S. |
| PES47 | 46 ± 1 | | 114 ± 11 | |
| PES47-pBABE-1 | 45 ± 1 | N.S. | 89 ± 4 | N.S. |
| PES47-pBABE-2 | 43 ± 1 | N.S. | 97 ± 3 | N.S. |
| PES47-DN-1 | 74 ± 1 | <0.0001 | 99 ± 1 | N.S. |
| PES47-DN-2 | 70 ± 2 | = 0.0001 | 98 ± 2 | N.S. |
| PES47-DN-3 | 71 ± 2 | <0.0001 | 86 ± 4 | N.S. |

N.S., not statistically significant.

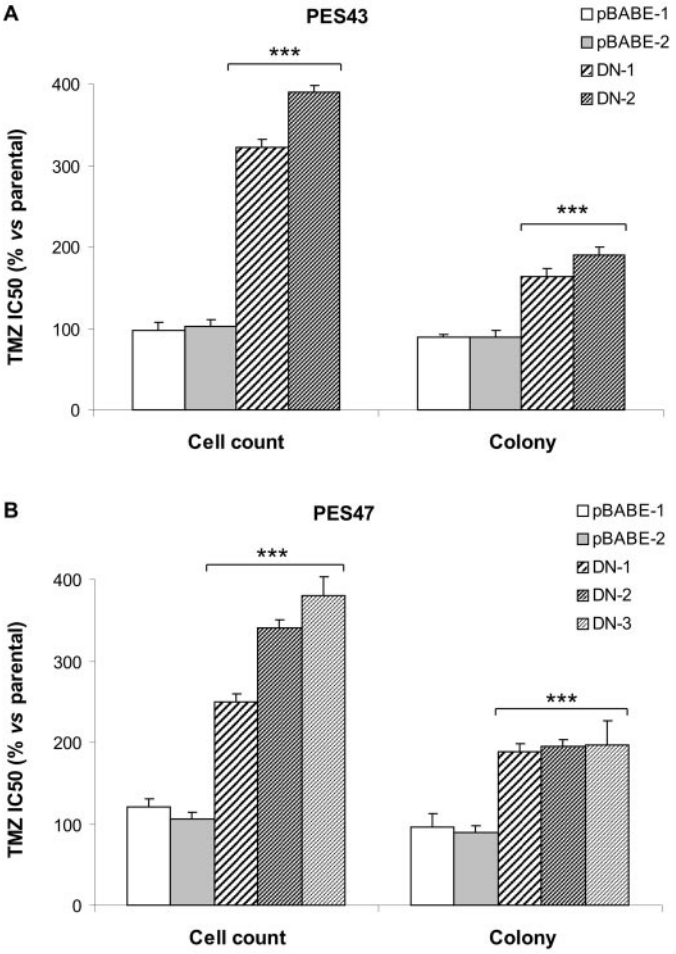


**Fig. 4.** Effect of telomerase inhibition by DN-hTERT expression on susceptibility of PES43 and PES47 melanoma clones to TMZ. The $IC_{50}$ values of pBABE and DN-hTERT clones are given as percentages of the $IC_{50}$ of parental melanoma PES43 or PES47 cell lines. Data are means from three independent experiments. The indicated means and the relative S.E. (bars) were calculated after angular transformation of the percentage values. Statistical analysis was performed according to Student's *t* test. Differences between parental line and DN-hTERT clones were statistically significant (***, $P < 0.001$), whereas differences between parental and pBABE clones were not statistically significant.

## Discussion

Overexpression of telomerase has been involved in resistance of tumor cells to chemotherapy. In the present study, we have investigated the role of telomerase expression in chemosensitivity of melanoma cells to TMZ, a novel agent with promising antitumor activity against metastatic melanoma and other incurable forms of cancer. We also assessed the potential influence of antitelomerase strategies aimed at reducing tumor growth on susceptibility of melanoma cells to TMZ and to other antineoplastic agents used for the treatment of this malignancy. In this study, we demonstrated for the first time that although basal telomerase expression did

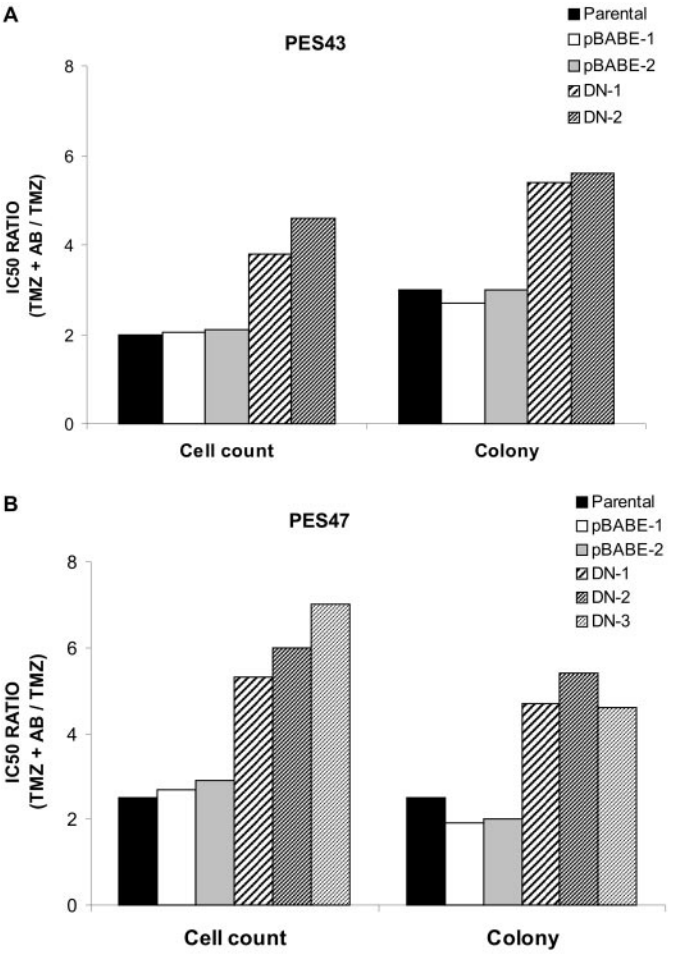


**Fig. 5.** Enhancement of the antiproliferative effect of TMZ mediated by PARP inhibitor in parental or transfected melanoma clones. The increase of growth inhibition induced by TMZ + AB in parental, pBABE, or DN-hTERT clones is expressed as ratio between TMZ + AB $IC_{50}$ and TMZ $IC_{50}$. Data are representative of one of three independent experiments with similar results.

not affect susceptibility of melanoma cells to TMZ, inhibition of telomerase activity resulted in reduced growth rate and increased resistance to TMZ. Of note was that association with a PARP inhibitor restored susceptibility to TMZ in telomerase-suppressed and slowing proliferating melanoma cells.

In total, six melanoma cell lines were characterized for telomerase activity and DNA repair enzymes, and three patterns of expression were observed (Table 2). CIMA62 cells were endowed with low telomerase activity, undetectable AGT levels, and higher susceptibility to TMZ with respect to CIMA73 cells. On the other hand, the telomerase-deficient MAS51 line showed lower susceptibility to TMZ compared with the M14 cell line. This is attributable, at least in part, to the higher AGT activity detected in MAS51 cells with respect to the parental line. Finally, PES43 and PES47, which expressed comparable AGT activity and growth rate but different telomerase levels, possessed sensitivity similar to that of TMZ.

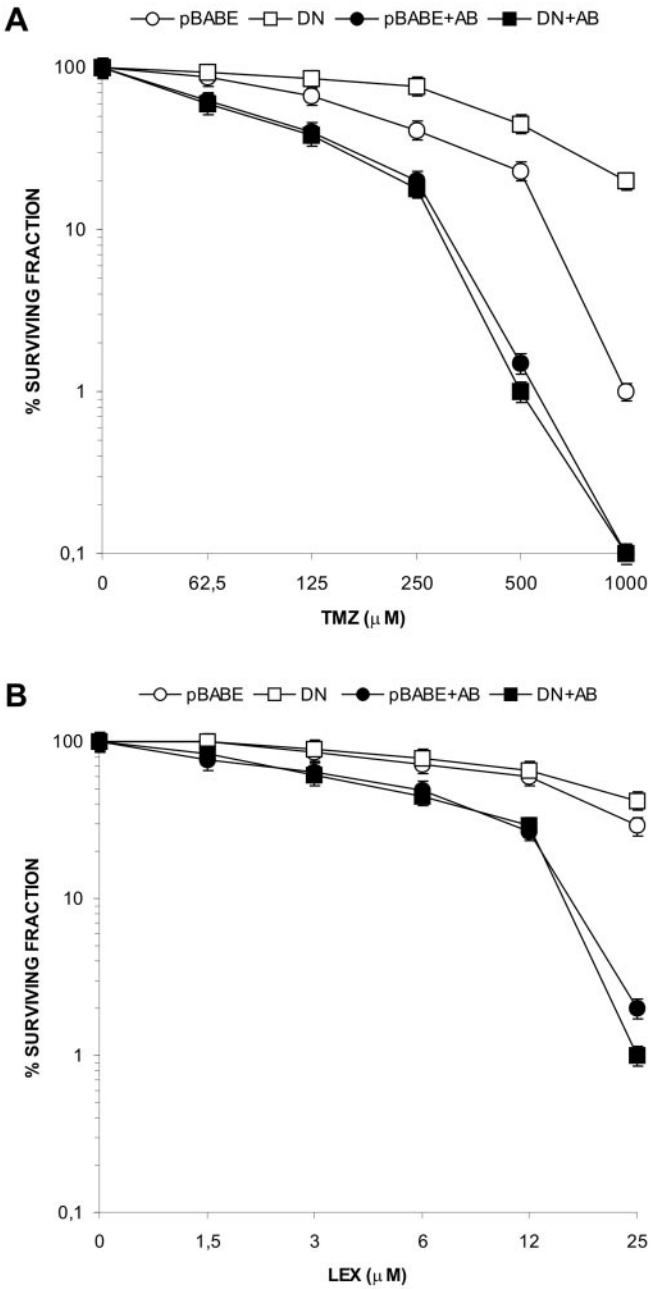


**Fig. 6.** Survival curves of PES47 pBABE and PES47 DN-hTERT clones treated with TMZ ± AB or with Lex ± AB. PES47 pBABE-2 and PES47 DN-2 cells were exposed to the indicated concentrations of TMZ ± AB (A) or Lex ± AB (B), and survival was assessed by colony-forming assay. Data are expressed as percentage of colonies formed by drug-treated cells with respect to untreated control cells and are representative of one of three experiments with comparable results. The indicated values are the mean of the percentages of survival calculated from three plates per group. Bars, S.E..

Thus, chemosensitivity to TMZ seemed to be dependent primarily on AGT activity rather than on telomerase levels. Furthermore, the lower sensitivity of PES lines with respect to MAS51 cells, which show comparable AGT activity, was not caused by lack of expression of MR components MLH1 and MSH2. These results suggest the existence of as-yet-uncharacterized mechanisms of resistance to TMZ.

Differences in basal telomerase expression in PES and CIMA lines were not associated with distinct patterns of proliferation rate. Only in MAS51 cells, which express low levels of *c-myc*, telomerase deficiency was accompanied by reduced growth with respect to *c-myc* proficient M14 parental line.

This said, the influence of telomerase inhibition was assessed in melanoma cell lines with similar growth rate and AGT activity. In PES cells, inhibition of telomerase by stable expression of a catalytically inactive, dominant-negative mutant of the enzyme resulted in reduced growth rate and increased resistance to both TMZ and the chloroethylating agent BCNU. However, susceptibility of DN-hTERT–expressing clones to the platinating compound CDDP was enhanced, whereas sensitivity to TAM did not change. AGT up-regulation or selection of cells with higher levels of AGT activity did not cause reduced response to TMZ and BCNU, because all the clones examined showed comparable levels of the DNA repair enzyme. In addition, AGT-deficient DN-hTERT clones derived from M14 cell line were 2- to 3-fold more resistant than telomerase-positive pBABE or parental control cells.

Increased resistance to TMZ, a wide-spectrum methylating agent, probably does not involve reduced sensitivity to the damage provoked by *N*3-methyladenine, which, unlike *N*7-methylguanine, is a highly toxic lesion (Engelward et al., 1998). In fact, susceptibility to the selective *N*3-adenine methylating compound Lex, which generates >90% *N*3-methyladenine adducts, was not affected by telomerase suppression. Moreover, all transfected clones expressed comparable levels of MPG activity (data not shown), which is known to play an important role in chemosensitivity to Lex (Engelward et al., 1998; Tentori et al., 2001a). These results suggest very different toxicity pathways for the different alkylating agents.

Inhibition of telomerase has been shown to induce a decrease in tumor cell proliferation that can be observed only when telomeres reach a critically short length (Hahn et al., 1999; Zhang et al., 1999). Therefore, it has been suggested that the delay in the appearance of the antiproliferative effect induced by telomerase inhibitors would necessarily require association of this therapeutic approach with other treatment modalities, such as chemotherapy. To date, however, few studies have investigated the association of antitelomerase therapies with chemotherapy and, for some antitu-

mor drugs, results seem to be controversial. In selected experimental models, telomerase inhibition resulted in increased sensitivity only to certain antitumor drugs. For instance, ribozyme cleavage of hTERT mRNA rendered breast cancer cells more susceptible to the topoisomerase II inhibitor etoposide and to the intercalating DNA damaging agent doxorubicin (Ludwig et al., 2001). Conversely, inhibition of telomerase did not affect the response of breast cancer cells to CDDP (Ludwig et al., 2001). Malignant glioblastoma cells could instead be sensitized to CDDP by transfection of an antisense human telomerase (Kondo et al., 1998, 2001). Finally, neoplastic cells from telomerase RNA-null mice were more susceptible to doxorubicin, but not to CDDP, etoposide, and the antimetabolite 5-fluorouracil with respect to their telomerase-positive counterparts (Lee et al., 2001).

In PES melanoma cell lines, expression of DN-hTERT, which resulted in a profound reduction of telomerase activity, was not accompanied with progressive telomere shortening and subsequent increased apoptosis or growth arrest, as observed in other experimental models (Hahn et al., 1999; Zhang et al., 1999). However, the growth rate of DN-hTERT clones was significantly reduced with respect to their telomerase-expressing counterparts (Table 3).

Reduced melanoma growth rate might, at least in part, account for the decreased susceptibility of melanoma clones expressing DN-hTERT to TMZ. Indeed, we demonstrated previously that both cytotoxic and growth inhibitory effects of TMZ are more evident in actively proliferating cells than in resting lymphocytes or growth-arrested tumor cells (Tentori 2001b, 2002b). Actually, DNA damage (i.e., unrepaired $O^6$-methylguanine adducts) requires DNA synthesis and occurs during the second cycle of replication, when thymine rather than cytosine is incorporated opposite the methylated base. Only at this point is the MR system triggered by the G:T

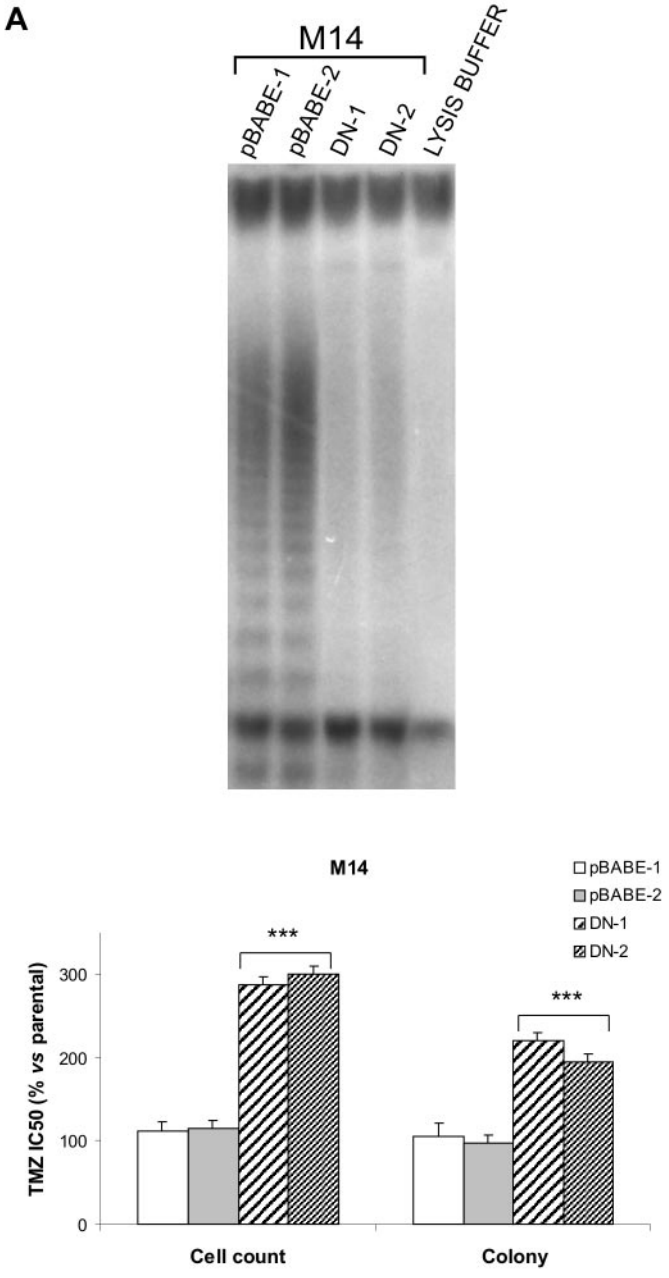


**Fig. 7.** Influence of DN-hTERT expression on M14 chemosensitivity to TMZ. A, analysis of telomerase activity in clones derived from melanoma M14 cell line infected with control vector (pBABE) or with DN-hTERT virus (DN). Telomerase activity was tested by TRAP assay using 100 ng of proteins. Negative control, cell extract was replaced by an equal volume of lysis buffer. B, the $IC_{50s}$ of pBABE and DN-hTERT clones are given as percentages of the $IC_{50}$ of parental melanoma M14 cell line. Data are means from three independent experiments. The indicated means and the relative S.E. (bars) were calculated after angular transformation of the percentage values. Statistical analysis was performed according to Student's $t$ test. Differences between parental line and DN-hTERT clones were statistically significant (***, $P < 0.001$), whereas differences between parental and pBABE clones were not statistically significant.

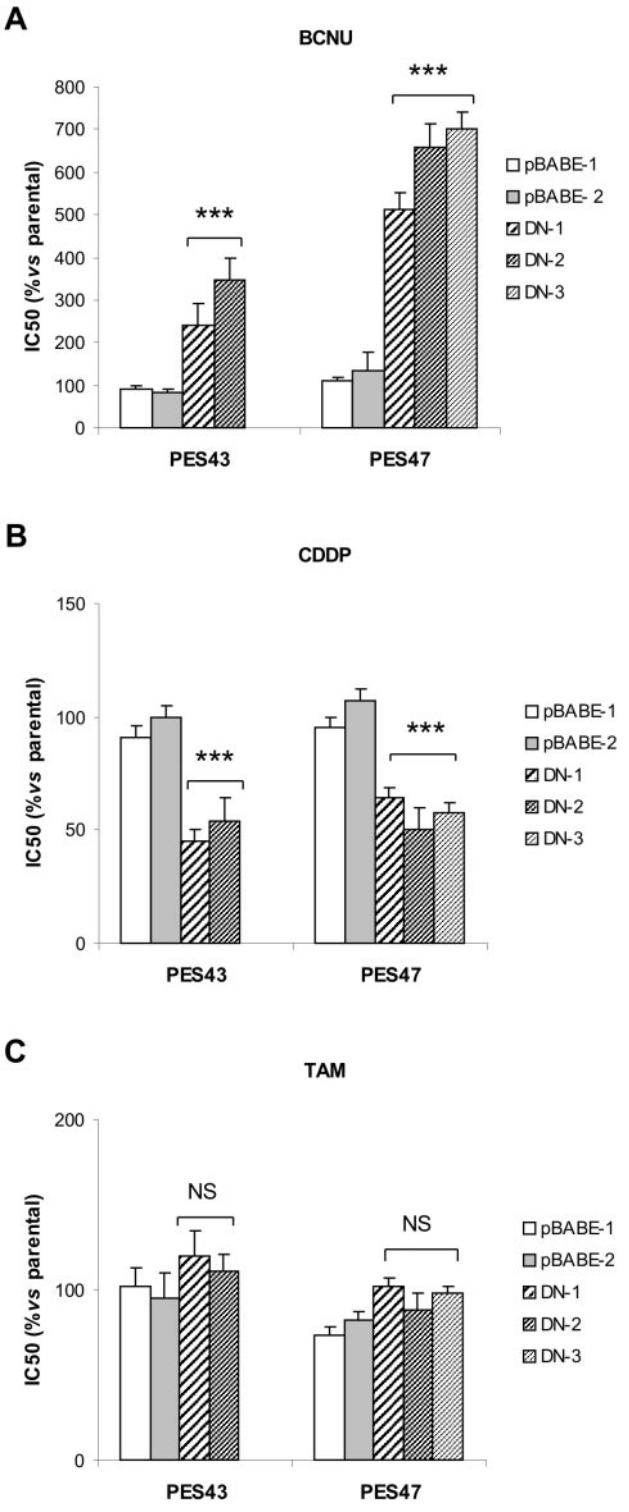


**Fig. 8.** Effect of telomerase inhibition by DN-hTERT expression on susceptibility of PES43 and PES47 melanoma cells to BCNU, CDDP, and TAM. $IC_{50}$ values of pBABE and DN-hTERT clones are given as percentages of the $IC_{50}$ of parental melanoma PES43 or PES47 cell lines. CDDP $IC_{50}$ in parental PES43 and PES47 lines were 1.1 ± 0.1 and 1.4 ± 0.1 $\mu$M, respectively. TAM $IC_{50}$ in parental PES43 and PES47 were 3.5 ± 0.3 and 4 ± 0.1 $\mu$M, respectively. Data are means from two independent experiments (three plates per group); bars, S.E. Differences between parental line and DN-hTERT clones treated with BCNU or CDDP were statistically significant (***, $P < 0.001$), whereas differences between parental and DN-hTERT clones treated with TAM were not statistically significant. Differences between parental and pBABE clones treated with BCNU, CDDP, or TAM were not statistically significant (NS).

mispairs, because MR exclusively removes thymine, which is inserted again during repair DNA synthesis. The futile cycles of MR intervention lead to growth arrest and/or apoptosis induction (D'Atri et al., 1998; Hirose et al., 2001). Thus, the increased doubling time observed in DN-hTERT transfectants might delay MR intervention and reduce the extent of growth inhibition induced by TMZ. In the case of BCNU, even if this agent is capable of alkylating DNA of nondividing cells, toxicity is markedly enhanced in rapidly proliferating tissues. Moreover, because of the lengthening of cell cycle duration occurring in telomerase-inhibited clones, the AGT enzyme might have more time to remove the chloroethyl adduct before the next cell division.

Interestingly, PARP inhibition restored sensitivity of DN-hTERT clones to TMZ. We previously demonstrated that the efficacy of TMZ and PARP inhibitor treatment does not require active cell proliferation (Tentori et al., 2001b, 2002b). In fact, in this case DNA damage derives from the interruption of *N*-methylpurine repair by the BER system and becomes evident during the first cycle of DNA duplication. Because DN-hTERT clones were more resistant to TMZ, the enhancement in growth inhibition induced by the PARP inhibitor was more pronounced in these cells than in parental or pBABE control cells. However, in parental or pBABE cells and in DN-hTERT clones, the average TMZ + AB $IC_{50}$ was similar, suggesting that DNA damage induced by drug combination was not affected by telomerase suppression.

The results obtained with CDDP in our model system do not agree with previous findings, which suggested that attenuation of telomerase did not alter the chemosensitivity profile of melanoma cells to a number of platinum compounds (Folini et al., 2000). Actually, Folini et al. (2000) also observed no changes in tumor cell response to topoisomerase I inhibitors and the microtubule poisons taxanes. Suppression of telomerase was achieved by hammerhead ribozyme targeting of the RNA component rather than by disruption of the catalytic subunit function, as accomplished in the present study. This might account for the different impairment of growth rate observed by these authors compared with that detected in our experimental model using DN-hTERT expression. Regardless of whether telomerase inhibition results in telomere shortening, the increase in doubling times shown in the present study with the melanoma DN-hTERT transfectants was at least 2-fold higher than that reported for ribozyme transfectants. Thus, the more pronounced reduction of growth rate might contribute to enhance the antiproliferative effects of CDDP.

In conclusion, our results indicate that a rational selection of antitumor agent(s) must be made when antitelomerase therapy is combined with chemotherapy to avoid possible antagonist effects on the activity of DNA damaging agents such as TMZ or BCNU, which might be less effective in tumor cells with reduced growth rate. Moreover, the data presented here demonstrate that PARP inhibitor enhances susceptibility of melanoma cells to TMZ, even when combined with telomerase inhibition, suggesting that TMZ + PARP inhibitor can be an effective drug combination that is not sensitive to differences in tumor repair capacities or to tumor growth rates.

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**Address correspondence to:** Grazia Graziani, Department of Neuroscience, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy. E-mail: graziani@uniroma2.it