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On the significance of telomerase activity in human malignant glioma cells

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Abstract

Telomerase is critical for tumor cell immortalization and is a novel target for cancer chemotherapy. Here, we examined whether telomerase is expressed in glioma cell lines, whether telomerase activity is regulated by bcl-2 or p53, and whether telomerase activity predicts response to chemotherapy. Further, we characterized the effects of a candidate telomerase inhibitor, penclomedine, in glioma cells. All 12 human malignant glioma cell lines examined were telomerase positive. Telomerase activity was not modulated during cell cycle progression, did not correlate with p53 status or bcl-2 family protein expression, and did not predict drug sensitivity, except for an association with resistance to carmustine. Ectopic bcl-2 expression did not enhance telomerase activity. Wild-type p53 reduced telomerase activity in cell lines retaining p53 activity but not in p53-mutant cell lines. Penclomedine killed glioma cells via an apoptotic, but death receptor-, bcl-2- and caspase-independent pathway, but did not inhibit telomerase and did not act synergistically with cytotoxic drugs. We conclude that telomerase activity does not account for the differential chemosensitivity of human glioma cells and that penclomedine kills glioma cells via a telomerase-independent pathway.

Keywords: Glioma; Telomerase; Chemosensitivity; Penclomedine; p53

1. Introduction

Telomerase is a ribonucleoprotein that stabilizes chromosome by adding $(TTAGGG)_n$ repeats to the telomeric ends. Telomerase has become a new target for cancer therapy because telomerase activity may be an intrinsic feature of the immortality acquired by cancer cells (Kim et al., 1994; Bodnar et al., 1998; Counter et al., 1998). Correspondingly, the differentiation of cancer cells is associated with an inhibition of telomerase activity (Sharma et al., 1995). Recent analyses of telomerase-deficient mice indicate that loss of p53 attenuates the deleterious effects of telomerase deficiency, indicating that both events might cooperate in carcinogenesis (Chin et al., 1999). Yet, induction of telomerase activity per se does not confer a malignant phenotype on normal cells (Jiang et al., 1999). Telomerase activity is undetectable in most mature normal tissues but has been identified in many human cancers, including malignant gliomas (Langford et al., 1995; Chong et al., 1998; DeMasters et al., 1997; Le et al., 1998).

The assessment of telomerase activity has been proposed to predict chemosensitivity in various human cancers in vitro. Further, cytotoxic drugs such as cisplatin or the experimental agent, penclomedine, may inhibit telomerase activity (Burger et al., 1997; Pandita et al., 1997; Ishibashi and Lippard, 1998; Wang et al., 1998). The inhibition of telomerase activity in U251MG glioma cells by antisense approaches resulted in differentiation and sensitization to cisplatin-induced apoptosis (Kondo et al., 1998a,b,c). Further, the inhibition of U87MG glioma cell growth mediated by antagonists of growth hormone-releasing hormone was associated with a decrease in telomerase activity as a result of down-regulation of human telomerase reverse transcriptase mRNA which encodes the telomerase catalytic subunit (Kiaris and Schally, 1999).

In the present study, we sought to investigate the role of telomerase in the malignant phenotype of malignant gliomas and its significance as a possible target for glioma

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therapy. To this end, we examined whether telomerase is expressed by a panel of 12 previously characterized human glioma cell lines (Weller et al., 1998). Further, given published data on the regulation of telomerase activity by bcl-2 (Mandal and Kumar, 1997) or p53 (Brown et al., 1998; Milas et al., 1998; Roos et al., 1998; Wu et al., 1999), we examined the impact of these gene products on telomerase activity. Using previously published data on chemosensitivity patterns in these cell lines (Weller et al., 1998), we investigated whether telomerase activity predicts the response to chemotherapy. Further, we characterized the effects of the candidate telomerase inhibitor, penclomedine (Pandita et al., 1997), on telomerase activity and chemosensitivity in glioma cells.

2. Materials and methods

2.1. Chemicals, cell lines and cell culture

Penclomedine was kindly provided by Dr. Tej K. Pandita (Houston, TX) or the Developmental. Therapeutics Program (National Cancer Institute, National Institute of Health, Bethesda, MD). G418 was purchased from Gibco (Basel, Switzerland). Hygromycin B, streptavidin alkaline phosphatase conjugate, 4-nitrobule tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Boehringer (Mannheim, Germany). Avidin/biotin peroxidase complex came from Vector (Burlingame, CA). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) was purchased from Molecular Probes (Eugene, OR). Vincristine and etoposide were provided by Bristol-Myers Squibb (Princeton, NJ). Lomustine was provided by Medac (Hamburg, Germany). Temozolomide was provided by Schering-Plough Research Institute (Kenilworth, NJ). Topotecan was obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). *N*-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (DEVD-amc) were obtained from Bachem (Heidelberg, Germany). L-Buthionine-(*S*,*R*)-sulfoximine, *N*-acetylcysteine, cytarabine, *N*-*tert*-butyl-α-phenylnitrone and all other chemicals, unless indicated otherwise, were obtained from Sigma (St. Louis, MO). CD95 ligand was obtained using supernatants from previously described Neuro-2A cells engineered to produce soluble murine CD95 ligand (Rensing-Ehl et al., 1995). Human glioma cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 1% glutamine and antibiotics. These cell lines have been characterized in previous studies (Weller et al., 1998). Glioma cells engineered to express high levels of murine bcl-2 (T98G, LN-229, LN-18) have been described (Weller et al., 1995). The generation of LN-18 and LN-229 cells engineered to express the murine temperature-sensitive p53 mutant val^{135} has also been reported (Trepel et al., 1998). U87MG and LN-308 transfectants stably expressing $p53val^{135}$ were obtained by lipofection with PrimeFector (EquiBio, Kent, England) using the $p53val^{135}$ hygro vector. Cells were selected for 3 weeks after transfection. Expression of the transfected p53 gene was confirmed by immunoblot analysis. Pooled transfected cells were compared with neo (bcl-2) or hygro (p53) control cells, which were transfected with the respective empty vectors lacking a cDNA insert. Death ligand (CD95L/Apo2L)-resistant LN-18 cells, and LN-18 and LN-229 cells engineered to express the viral caspase inhibitor, crm-A, and puromycin resistant control transfectants (puro) have been described (Glaser et al., 1999; Wagenknecht et al., 1998). Primary astrocytes were prepared from 7-day-old rats. The cerebrum was rinsed twice with phosphate-buffered saline (PBS) and cut into small pieces. After incubation in serum-free medium containing 20% trypsin, the cell suspension was seeded in 75-cm^2 culture flasks in DMEM containing 20% fetal calf serum, 1% glutamine and antibiotics. The medium was changed to standard cell medium as described above after 5 days. Second passage cells were used for all experiments. Cell cycle analysis was performed by flow cytometry (Hueber et al., 1998). The formation of reactive oxygen species was detected using H_2-DCFDA fluorescein (Reber et al., 1998). Caspase activity was measured by conversion of the fluorescent substrate, DEVD-amc, as previously described (Wagenknecht et al., 1998). Drug cytotoxicity studies were performed in 96-well plates. Glioma cell survival or proliferation was assessed by crystal violet staining at the indicated times after drug exposure (Weller et al., 1998) To confirm that the crystal violet staining data represented actual cell death rather than growth inhibition, lactic acid dehydrogenase (LDH) release was assessed using the LDH Cytotoxicity Kit (Boehringer). The cells were treated for 24 h with increasing concentrations of penclomedine. Triton X-100 (2%) was used to determine the maximal LDH release. Electron microscopic analysis was performed as described (Glaser et al., 1999).

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of mRNA encoding the catalytic subunit of human telomerase reverse transcriptase was assessed by RT-PCR. Total RNA was isolated using the RNeasy Mini Kit (Quiagen, Hilden, Germany). cDNA was prepared using random hexamer primers and Superscript II (Gibco, Gaithersburg, MD). PCR primers were obtained from MWG Biotech (Ebersberg, Germany) (Kiaris and Schally, 1999). The primer sequences were as follows: telomerase sense (nucleotides 1784–1803), 5′-CGGAAGAGTGTCTGGAGCAA-3′, telomerase antisense (nucleotides 1928–1910), 5′-GGATGAAGTGGAGTCTGG-3′, yielding a PCR product of 145 bp; human β-actin sense (nucleotides 26–46), 5′-TGTTTGAGACCTTCAACACCC-3′, human β-actin antisense (nucleotides 896–877), 5′-AGCACTGTGT-

TGGCGTACAG-3′, yielding a PCR product of 871 bp. The PCR conditions for telomerase cDNA were: 5 min 94°C; 40 × 30 s/94°C, 30 s/62°C, 30 s/72°C; 7 min 72°C. This was followed by a pseudo-nested PCR with the same telomerase primer pair: 1/50 PCR product; 5 min 94°C; 30 × 30 s/94°C, 30 s/62°C, 30 s/72°C; 7 min 72°C. The annealing temperature was 55°C and 30 cycles for the β-actin control PCR. The PCR products were run

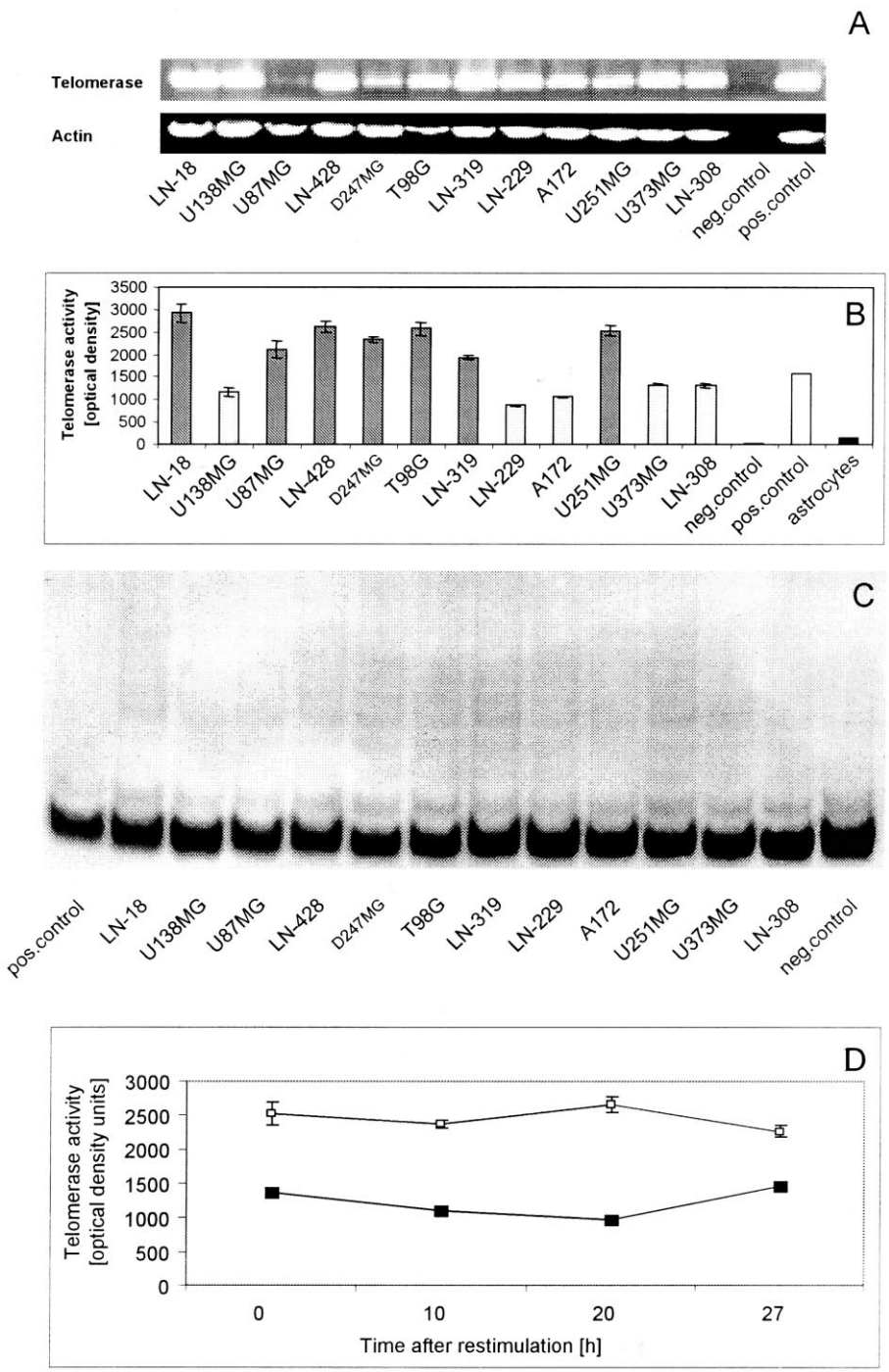


Fig. 1. Telomerase activity in human malignant glioma cell lines. (A) RT-PCR for the catalytic subunit of telomerase was performed as described in Section 2. Amplified cDNA fragments were visualized by ethidium bromide staining after separation on a 2% agarose gel. (B) Telomerase activity was determined by PCR-ELISA. Data are expressed as mean OD units and S.E.M. ($n = 3$). The human telomerase-positive embryonic kidney cell line 293 was used as a positive control. LN-18 cell lysates were heat-inactivated (10 min/94°C) prior to primer elongation and used as a negative control. (C) The specific formation of a 6-bp laddering pattern corresponding to telomerase activity was ascertained by gel electropheresis and Southern blot analysis. (D) LN-229 (filled squares) or T98G (open squares) cells were serum-deprived for 60 h and subsequently restimulated with serum-containing medium for 10, 20 or 27 h. Telomerase activity was measured at the indicated time points. Data are expressed as mean OD units and S.E.M. ($n = 3$).

on 2% agarose and visualized by ethidium bromide staining.

2.3. Telomerase PCR-enzyme-linked immunosorbent assay (ELISA) and Southern blot analysis

Telomerase activity was determined using the Telomerase PCR-ELISA kit (Boehringer) according to the manufacturer's manual. This assay is based on the specific amplification of telomerase-mediated elongation products combined with nonradioactive detection using an ELISA protocol and is as such a more sensitive approach to the detection of telomerase activity than the original assay (Kim et al., 1994). Briefly, the cells were lysed and the lysates were adjusted to 0.5 μg protein/μl. Negative controls were obtained by heat inactivation of telomerase at 94°C for 10 min. Telomeric repeat amplification was performed using 2 μl of lysate. The elongation of the primers by telomerase took place at 25°C (30 min). Telomerase was inactivated by two cycles of 94°C/5 min. The PCR conditions were: 30 × 30 s/94°C, 30 s/50°C, 90 s/72°C, followed by primer elongation at 72°C for 10 min. Five microliters of PCR product was denatured and hybridized to a digoxigenin-labeled telomeric repeat-specific detection probe for 2 h at 37°C on a shaker. The resulting product was immobilized via the biotin-labeled primer to a streptavidin-coated microtiter plate. Immobilized PCR product was detected with anti-digoxigenin peroxidase using 3,3′,5,5′-tetramethyl benzidine as a substrate. Color development was stopped after 15 min. The absorbance of the samples was measured at 450 nm (with a reference wavelength of 650 nm) using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA). To verify the telomerase-specific 6-bp ladder formation, Southern blot analysis was performed, using 35 μl of the PCR product in a 12.5% polyacrylamide gel in 0.5 × Tris/boric acid/EDTA (TBE) electrophoresis buffer. The gel was run for 6.5 h at 100 V. DNA was transferred to a positively charged nylon membrane (Hybond N+, Amersham, Braunschweig, Germany) by capillary blotting overnight. After crosslinking at 1.5 J/cm^2 in a UV-crosslinker (Stratagene, La Jolla, CA), the membrane was washed once in PBS/Tween 0.05%. Unspecific binding sites were blocked with blocking buffer (Tris–HCl, 10 mM; NaCl, 150 mM; Tween 20, 0.1%; skimmed milk, 5%; bovine serum albumin, 2%; sodium azide, 0.01%; pH = 7.4–7.6) for 6 h. After the membrane was washed in PBS/Tween 20 0.05%, 20 ml of avidin/biotin complex (ABC) reagent (Vectastain, Vector) was added and incubated for 1 h. The membrane was washed twice in PBS/Tween 0.05%, and the bands were visualized using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

2.4. Statistical analysis

Data are expressed as mean values and S.D. or S.E.M. from experiments performed at least three times with similar results. Telomerase activity in bcl-2- or p53-transfected cell lines was compared with control-transfected (neo, hygro) cell lines by *t*-test. To investigate the influence of genetic status and protein expression on telomerase activity, the telomerase activity of the groups (for example, cell lines with wild-type p53 status vs. cell lines with mutant p53 status) was compared by *t*-test, and *P*-values < 0.05 were considered significant. To investigate the influence of telomerase activity on sensitivity to cytotoxic drugs, the EC_{50} values for the cytotoxic drugs in the cell lines with a low telomerase activity were compared with those in cell lines with a high telomerase activity by *t*-test. In addition, telomerase activity was correlated with EC_{50} values by Pearson's product moment correlation. Possible synergy was examined by the isobologram method (Berenbaum, 1981). LN-18 and LN-229 cells were cotreated with penclomedine and different cytotoxic agents. Each concentration of a drug was coadministered with a concentration of another drug, resulting in several different EC_{50} values for each drug combination. A graph, showing the concentration of penclomedine relative to the EC_{50} of penclomedine alone on the abscissa and the corresponding concentration of another drug relative to its EC_{50} on the ordinate, can be used to determine a synergistic, antagonistic or independent manner of action of the two drugs. Synergy is assumed if the graph plotted from the EC_{50} values of the coincubation is below the theoretical graph for an independent mechanism ($f(x) = 1 - x$).

3. Results

3.1. Determination of telomerase activity and correlation with drug sensitivity

All cell lines expressed mRNA for the catalytic subunit of human telomerase (Fig. 1A). Optical densities (OD),

Table 1
Correlation of telomerase activity and chemosensitivity in human malignant glioma cell lines. Mean EC_{50} values (μM) and S.E.M. for various drugs in cytotoxic cell death and modified clonogenic cell death assays in a group of seven cell lines with high telomerase activity (2485 ± 300) and a group of five cell lines with low telomerase activity (1310 ± 393) (see Fig. 1) were derived from previously published data (Weller et al., 1998) and compared by *t*-test

	Cytotoxic cell death		Clonogenic cell death	
	Telomerase activity		Telomerase activity	
	High	Low	High	Low
Carmustine	343.6 ± 157.4	257.0 ± 101.9	140.0 ± 48.8[a]	41.0 ± 7.8[a]
Vincristine	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.5 ± 0.2
Cytarabine	350.1 ± 396.6	411.4 ± 332.2	36.0 ± 39.0	16.9 ± 10.0
Teniposide	2.4 ± 1.8	3.2 ± 1.5	0.9 ± 0.4	1.1 ± 0.5
Doxorubicin	0.5 ± 0.3	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
Camptothecin	0.6 ± 0.7	0.4 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
β-Lapachone	1.0 ± 0.3	1.1 ± 0.3	0.6 ± 0.2	0.8 ± 0.4
Gemcitabine	7.2 ± 4.9	0.5 ± 0.3	2.0 ± 1.0	1.0 ± 0.7

[a] $P < 0.05$.

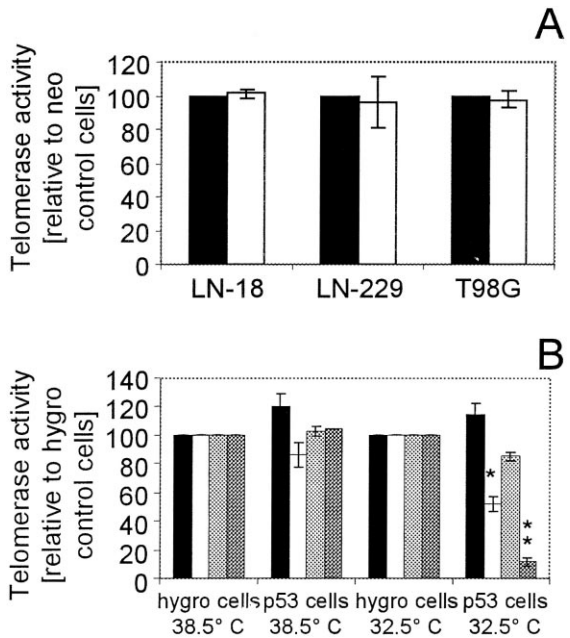


Fig. 2. Modulation of telomerase activity by bcl-2 and p53 gene transfer. (A) Glioma cell lines LN-18, LN-229 and T98G were transfected with the BMG-neo control plasmid (closed bars) or the BMG-bcl-2 (open bars) expression plasmid (Weller et al., 1995) and assayed for telomerase activity. Data are expressed as mean percentages of change of telomerase activity relative to neo control cells and S.E.M. ($n = 3$). (B) Hygro control cells or p53val^{135} transfectants of the LN-18 (closed bars), LN-229 (open bars), LN-308 (light gray bars) or U87MG (dark gray bars) cell lines were assayed for telomerase activity. The figure shows relative changes in telomerase activity at 38.5°C (mutant p53) or after a shift to 32.5°C (wild-type p53) for 48 h in p53val^{135}-transfected cells compared with control cells (mean and S.E.M., $n = 4$) ($^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test). U87MG control cells, but not the other cell lines, showed a marked increase in telomerase activity when switched to 32.5°C, compared with U87MG cells maintained at 38.5°C.

which reflect telomerase activity, ranged from 0.8 to 3 in the PCR-ELISA (Fig. 1B). None of the glioma cell lines was negative for telomerase activity since absorbance values exceeding 0.2 are regarded as positive in this assay. Heat-inactivated negative controls did not show any telomerase activity. Despite low telomerase mRNA expression, U87MG displayed significant telomerase activity in the PCR-ELISA (Fig. 1A and B). Primary rat 7-day postnatal astrocytes were devoid of telomerase activity. The formation of typical, telomerase-mediated 6-nucleotide ladders was confirmed by Southern blot analysis of the PCR products (Fig. 1C). Cell lines with high telomerase activity by PCR-ELISA, such as LN-18 or U251MG, also showed prominent laddering on gel electrophoresis. Telomerase activity has been proposed to be regulated during cell cycle progression (Zhu et al., 1999). Using a previously established paradigm of serum deprivation with synchronous G0/1 arrest and S phase re-entry (Hueber et al., 1998), we found no significant change from baseline activity in T98G or LN-229 cells at any time point studied (*t*-test, $P > 0.05$) (Fig. 1D).

The cell lines have previously been characterized with respect to genetic alterations and sensitivity to cancer chemotherapy (Weller et al., 1998). When the cell lines were divided into groups with low (U138MG, LN-229, A172, U373MG, LN-308; 1310 ± 393 U/ml) or high (LN-18, U87MG, LN-428, D247MG, T98G, LN-319, U251MG; 2485 ± 300 U/ml) telomerase activity, cell lines with low telomerase activity were significantly more sensitive to carmustine in a clonogenic cell death assay. No associations between telomerase activity and sensitivity to vincristine, cytarabine, teniposide, doxorubicine, camptothecin, β-lapachone or gemcitabine became apparent (Table 1).

3.2. No modulation of telomerase activity by bcl-2 gene transfer

There was no correlation between telomerase activity (Fig. 1) and the level of expression of bcl-2, bcl-xl, bcl-xs,

Table 2
p53 status, telomerase activity, and sensitivity to penclomedine cytotoxicity in human malignant glioma cell lines

	p53 status	Telomerace activity [optical density units]	EC_{50} for cytotoxic cell death [μM]	EC_{50} for clonogenic cell death [μM]
LN-18	mutant	2917 ± 215.5	56.5 ± 4.2	46.8 ± 2.7
U138MG	mutant	1168 ± 88.6	70.6 ± 2.1	41.7 ± 5.0
U87MG	wild-type	2116 ± 188.7	77.2 ± 4.2	36.9 ± 0.5
LN-428	mutant	2620 ± 135.5	82.6 ± 6.1	54.4 ± 0.7
D247MG	wild-type	2330 ± 62.4	85.3 ± 3.8	60.1 ± 6.4
T98G	mutant	2576 ± 142.3	68.5 ± 2.9	69.1 ± 9.9
LN-319	mutant	1937 ± 55.1	70.3 ± 7.8	53.5 ± 1.7
LN-229	wild-type	873 ± 8.4	95.5 ± 13.1	58.9 ± 3.0
A172	wild-type	1059 ± 13.6	98.4 ± 7.0	41.3 ± 2.6
U251MG	mutant	2526 ± 110.0	70.3 ± 4.5	55.3 ± 4.6
U373MG	mutant	1336 ± 25.3	76.9 ± 3.5	58.0 ± 6.1
LN-308	deleted	1311 ± 53.7	88.3 ± 5.4	38.3 ± 0.5

The table provides p53 status determined as detailed in Section 2, telomerase activity (see Fig. 1A), and mean EC_{50} values (μM) and S.E.M. for penclomedine in acute cytotoxic and clonogenic cell death assays.

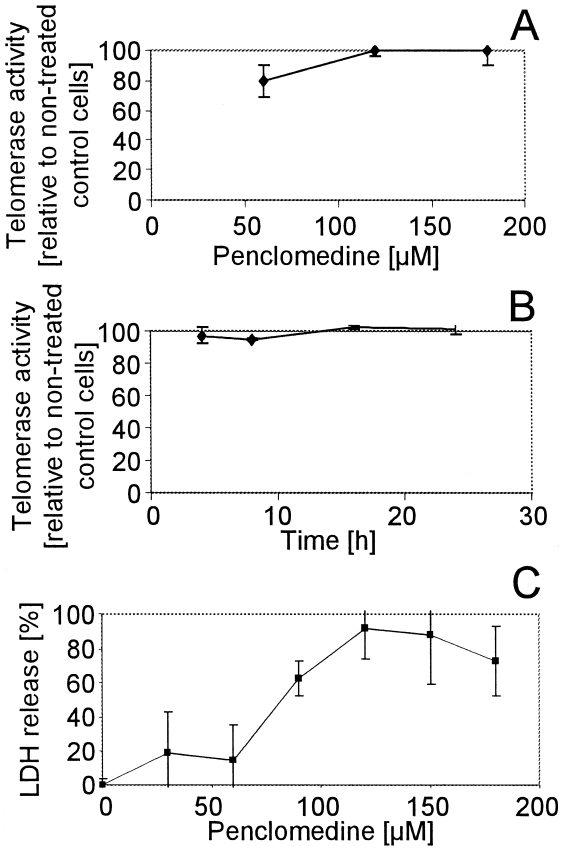


Fig. 3. No inhibition of telomerase activity by penclomedine. U251MG cells were treated with increasing concentrations of penclomedine for 4 h (A) or with 120 μM penclomedine for various lengths of time (B). Telomerase activity was measured as in Fig. 1B. In panel (C), penclomedine cytotoxicity was assessed by LDH release at 24 h.

mcl-1, bax or bad proteins, as determined by immunoblot analysis (Weller et al., 1998). Since bcl-2 gene transfer has been reported to result in an up-regulation of telomerase activity in HeLa cells (Mandal and Kumar, 1997), we next analyzed three glioma cell lines (LN-18, LN-229, T98G) engineered to express a murine bcl-2 transgene (Weller et al., 1995) for changes in telomerase activity. The functional anti-apoptotic activity of the bcl-2 transgene was verified by its inhibitory effect on CD95-mediated apoptosis. Ectopic bcl-2 expression did not affect telomerase activity in either of the three human glioma cell lines (*t*-test, $P < 0.05$) (Fig. 2A).

3.3. Modulation of telomerase activity by mutant or wild-type p53 gene transfer

Several studies have reported a relationship between p53 status and telomerase activity (Roos et al., 1998; Mukhopadhyay et al., 1998; Wu et al., 1999). The p53 status of the 12 cell lines examined here has been described earlier (Weller et al., 1998). Functional p53 activity was confirmed here by the demonstration of p53 and p21 protein accumulation in response to a defined stimulus of genotoxic stress, that is, exposure for 24 h to camptothecin at 2 μM (data not shown) (Table 2). We found no significant difference between cell lines with p53 wild-type activity (U87MG, D247MG, A172, LN-229; 1590 ± 740 OD units) and those with mutant or deleted genes (all other cell lines: 2050 ± 700 OD units). To further investigate the role of p53 in the regulation of telomerase activity, we transfected LN-18, LN-229, LN-308 and U87MG cells with the murine p53val^{135} plasmid, which encodes a temperature-sensitive p53 mutant that assumes mutant conformation at 38.5°C but exhibits wild-type properties at 32.5°C in human glioma cells (Trepel et al., 1998). p53val^{135}-transfected cells were compared with hygromycin-resistant control transfectants at 38.5°C (mutant p53) or

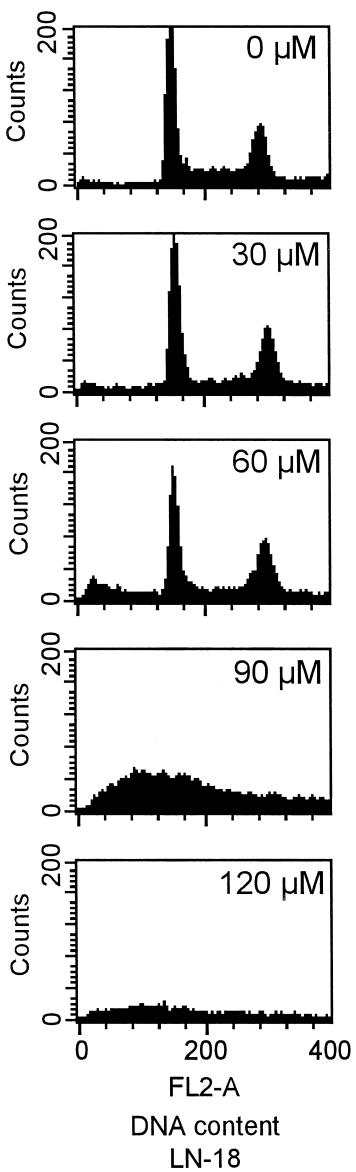


Fig. 4. Penclomedine does not induce specific changes in cell cycle distribution in human glioma cells. LN-18 cells were untreated or exposed to penclomedine at 30, 60, 90 or 120 μM for 24 h. Cell cycle analysis was performed by flow cytometry.

32.5°C (wild-type p53). Telomerase activity was determined at 38.5°C or after a shift to 32.5°C for 48 h. LN-18 cells, which have a mutant p53 background, and LN-308 cells, which have a p53 null background, showed no modulation of telomerase activity at either temperature (Fig. 2B). In contrast, the expression of wild-type p53 val^{135} in LN-229 and U87MG cells led to a reduction of telomerase activity to $52.1 \pm 5.3\%$ (mean and S.E.M.) in LN-229 and $11.63 \pm 3.1\%$ in U87MG cells whereas mutant p53 val^{135} (38.5°C) had no such effect.

3.4. Penclomedine induces apoptotic, death receptor-, bcl-2 and caspase-independent cell death in glioma cells, but does not inhibit telomerase

Penclomedine (NSC-338720) is an alkylating agent (Benvenuto et al., 1995) that has been reported to inhibit telomerase activity in human cervical carcinoma cells (HeLa) (Pandita et al., 1997). Therefore, we next assessed the modulation of telomerase activity by this drug in U251MG cells and the sensitivity of the 12 human glioma cell lines to penclomedine-induced cell death. U251MG cells were treated with increasing concentrations of penclomedine for 4 h, or at 120 μM for different lengths of time, and measured for telomerase activity. Telomerase activity was unaffected by penclomedine at concentrations up to 180 μM after a 4-h incubation and after treatment with 120 μM for up to 24 h (Fig. 3A and B). Penclomedine was uniformly active against the glioma cell lines, with EC_{50} values in the range of 50–100 μM in cytotoxic cell death assays and 30–60 μM in clonogenic cell death assays (Table 2). Maximal cytotoxicity was achieved after a 24-h treatment in the acute cytotoxicity assays. Prolonged exposure up to 72 h did not further enhance cytotoxicity. Penclomedine was also cytotoxic to rat early postnatal astrocytes (EC_{50}: 92.3 ± 18.5 μM), indicating that its activity is not restricted to the neoplastic phenotype

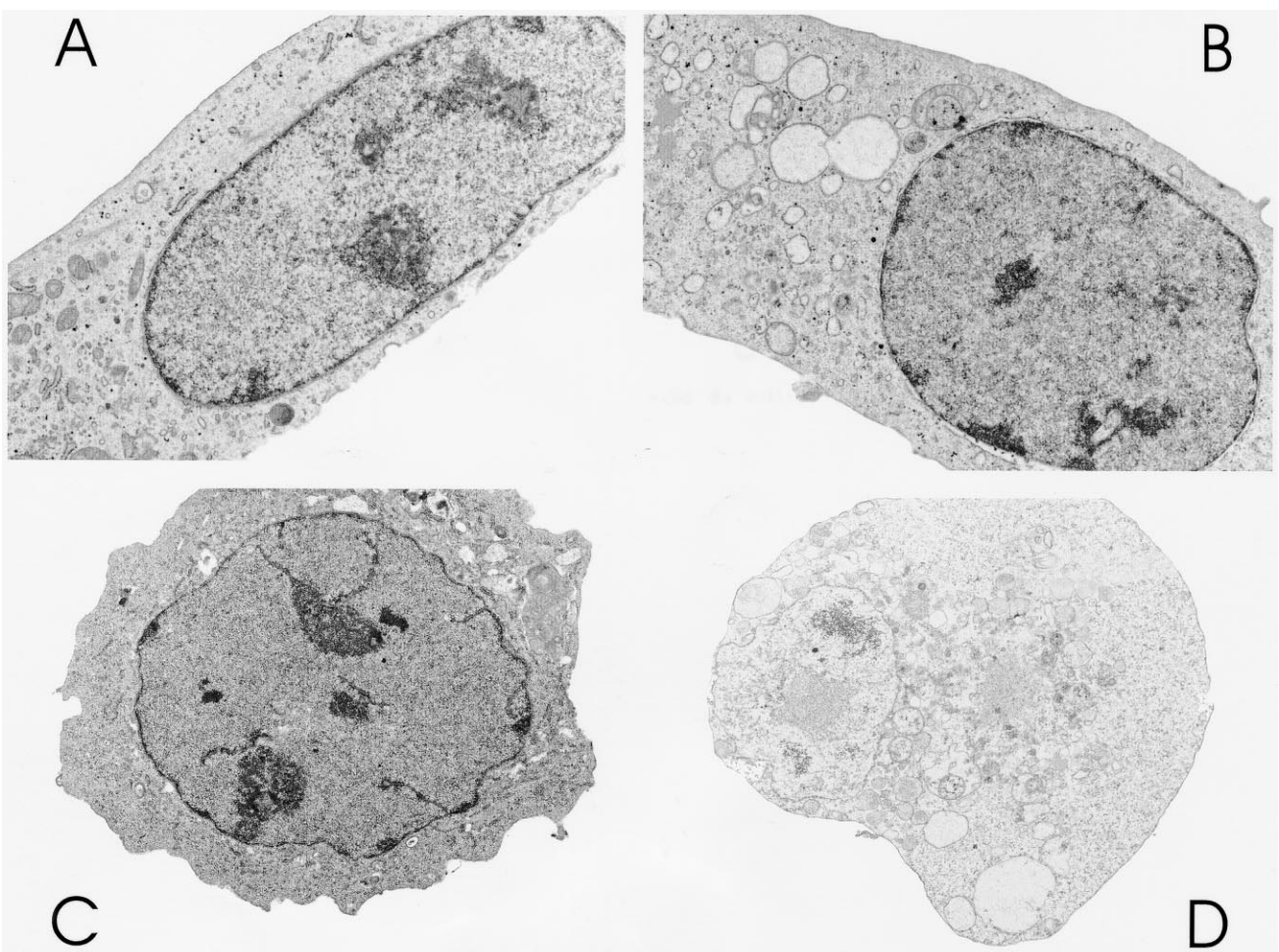


Fig. 5. Ultrastructural changes associated with penclomedine cytotoxicity in glioma cells. LN-18 cells were untreated (A) or treated with penclomedine (90 μM) for 6 h (B) or 24 h (C, D). Note cytoplasmic vacuoles and incipient condensation of chromatin in (B), blebbing, electron-dense cytoplasm and chromatin condensation in (C), and dominant cytoplasmic pathology in (D) (magnification: A, ×8752; B, ×9714; C, ×7223; D, ×7142, for details, see text).

of glioma cells. LDH release-based cytotoxicity assays performed in parallel with the determination of telomerase activity confirmed that cell death evolved in the absence of altered telomerase activity (Fig. 3C). There was also no inhibition of telomerase activity when penclomedine was added directly to the lystates (data not shown). As a positive control, we observed that the putative inducer of glioma cell differentiation, lovastatin (20 μM), reduced telomerase activity to 47 ± 3% ($n = 3$, mean and S.E.M.) in T98G cells. No specific change of cell cycle distribution assessed by flow cytometry was induced by penclomedine in LN-18 or LN-229 cells, except for an accumulation of dead cells at higher concentrations (Fig. 4).

Electron microscopy in LN-18 and LN-229 cells exposed to penclomedine at 30–150 μM for 6 or 24 h revealed that higher concentrations of penclomedine (90–120 μM) induced typical apoptotic changes of chromatin condensation and membrane blebbing within 6 h. In parallel, penclomedine induced prominent cytoplasmic vacuolization affecting the mitochondria and endoplasmic reticulum. These changes became more prominent at 24 h (Fig. 5). Further evidence to support that penclomedine does not kill glioma cells by inhibiting telomerase activity stems from the report that 60% of U251MG glioma cells treated with antisense to specifically inhibit telomerase activity did not undergo apoptosis until 30 doubling lines after treatment (Kondo et al., 1998b). The very narrow range of EC_{50} values for penclomedine for all 12 cell lines results a priori in the absence of a statistically significant interrelation not only of penclomedine cytotoxicity and telomerase activity but also of penclomedine cytotoxicity and p53 status, bcl-2 family protein expression or sensitivity to other cancer chemotherapy drugs. For illustration, the range of sensitivity, as determined by the ratio of highest over lowest EC_{50} values, was only 1.74 (A172, highest; LN-18, lowest) for penclomedine in acute cytotoxicity assays and 1.88 (T98G, highest; U87MG, lowest) in clonogenic cell death assays. In contrast, this ratio was between 4 for β-lapachone and 2000 for cytarabine in a previous study (Weller et al., 1998).

The next series of experiments was performed to elucidate the cellular events leading to penclomedine-induced cell death. We first asked whether reactive oxygen species were involved in the cytotoxic effects of penclomedine in LN-18 or LN-229 cells. To this end, we measured free radical formation after exposure to penclomedine and examined whether anti-oxidants inhibited penclomedine cytotoxicity. Penclomedine-induced cell death did not involve the formation of reactive oxygen species (Fig. 6A), was unaffected by *N-tert*-butyl-α-phenylnitrone or *N*-acetylcysteine (Fig. 6B) and was not enhanced by L-buthionine-[*S,R*]-sulfoximine-mediated glutathione depletion (Fig. 6C). The following experiments validated these data: ethacrynic acid and betulinic acid were used as a positive control for the formation of free radicals (Reber et al., 1998; Wick et al., 1999), betulinic acid cytotoxicity was

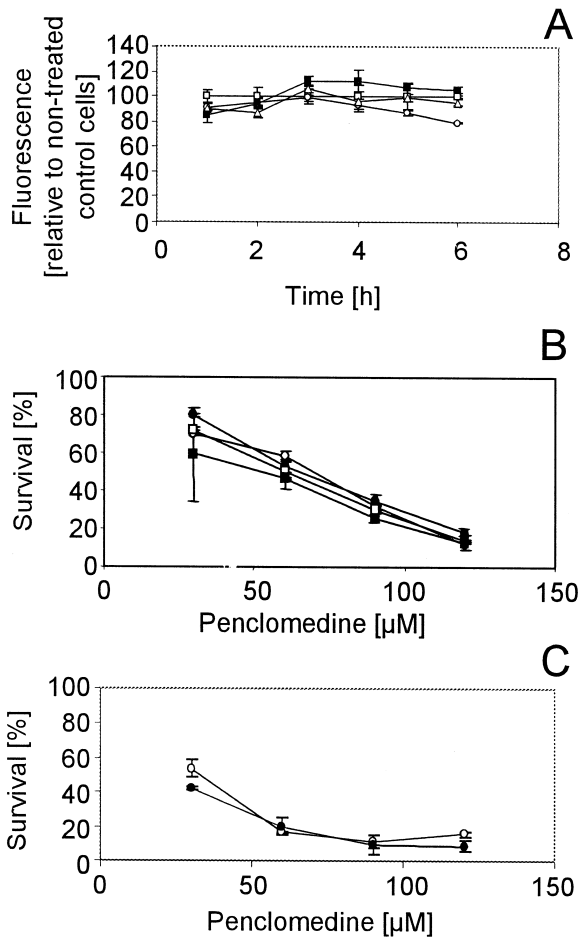
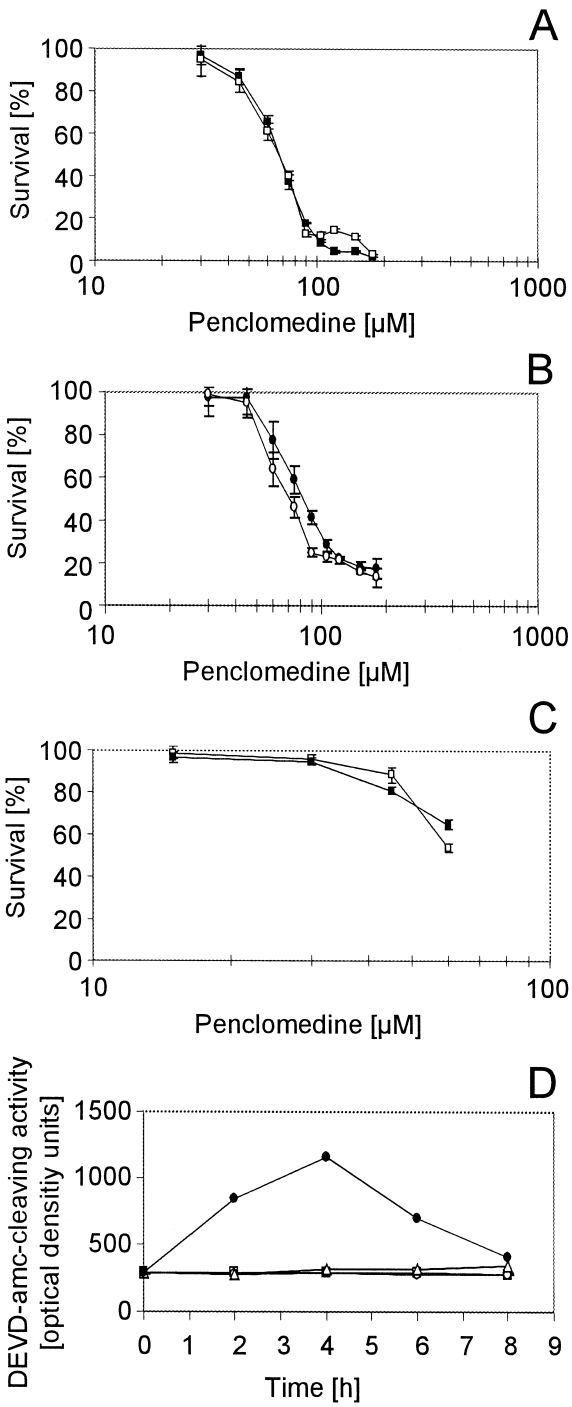


Fig. 6. Penclomedine does not induce the formation of ROS and is unaffected by anti-oxidants. (A) LN-18 cells were untreated (open squares) or treated with penclomedine at 30 (open triangles), 60 (open circles) or 90 μM (filled squares) for 1–6 h. The formation of Reactive Oxygen Species (ROS) was monitored by the addition of H_2-DCFDA (10 μM final concentration). Ethacrynic acid was used as a positive control (not shown). Data are expressed as mean relative fluorescence values and S.E.M. compared with untreated cells. (B) LN-18 were incubated with increasing concentrations of penclomedine in the absence (open circles or squares) or presence of *N-tert*-butyl-α-phenylnitrone (150 μM, closed circles) or *N*-acetylcysteine (100 μM, closed squares). (C) LN-18 cells were not pretreated (open circles) or preincubated with L-buthionine-[*S,R*]-sulfoximine (1 mM, closed circles) for 15 h, and subsequently treated with penclomedine for 24 h in the absence or presence of L-buthionine-[*S,R*]-sulfoximine. Survival was assessed by crystal violet staining (mean and S.E.M., $n = 3$).

used as a positive control for the protective effects of *N*-tert-butyl-α-phenylnitrone and *N*-acetylcysteine (Wick et al., 1999), L-buthionine-[*S,R*]-sulfoximine treatment lowered glutathione levels to approximately 20% (Reber et al., 1998), and treosulfan cytotoxicity was used as a positive control for the potentiation afforded by glutathione depletion (Reber et al., 1998).

Next, we asked whether endogenous death receptor/ligand interactions or caspases play a role in penclomedine cytotoxicity. LN-18 cells selected for resistance to cyto-

toxic cytokines, LN-18-R (Glaser et al., 1999), were as susceptible to penclomedine as the parent LN-18 cells (Fig. 7A). Also, LN-18 cells engineered to express the viral caspase 8 inhibitor, crm-A, were as susceptible to penclomedine as the puro control cells (Fig. 7B). In contrast, these cells are completely resistant to CD95L-induced apoptosis (Wagenknecht et al., 1998). zVAD-fmk, a broad-spectrum caspase inhibitor, did not inhibit penclomedine-induced cell death (Fig. 7C), suggesting that



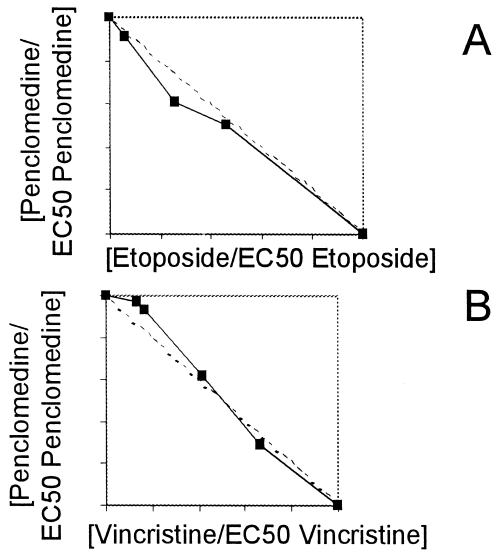


Fig. 8. Penclomedine-based combination chemotherapy in vitro: lack of synergy. LN-18 cells were cotreated with penclomedine and different concentrations of etoposide (A) or vincristine (B) for 72 h. Possible synergy was assessed by isobologram analysis. The dotted line represents the predicted graph in the absence of drug interaction.

neither death ligand/receptor interactions nor caspases mediate penclomedine-induced cell death. In accordance with these data, penclomedine did not induce DEVD-amc-cleaving caspase activity (Fig. 7D). To corroborate these findings, we confirmed that neither crm-A nor zVAD-fmk protected LN-229 cells from penclomedine cytotoxicity. Penclomedine cytotoxicity was also unaffected by the expression of bcl-2 or p53val[135] in mutant or wild-type cells (data not shown).

Finally, we investigated whether penclomedine modulated the efficacy of chemotherapy for glioma in vitro. To this end, we cotreated LN-18 and LN-229 cells with different concentrations of penclomedine, lomustine, topotecan, temozolomide, vincristine, etoposide or cytarabine for 24, 48 or 72 h. Isobologram analysis failed to reveal relevant synergy for any drug combination with penclomedine (Fig. 8).

Fig. 7. Penclomedine cytotoxicity does not involve death ligand/receptor interactions or caspase activation. (A) Parental LN-18 cells (filled squares) or CD95L/Apo2L-resistant LN-18 cells (open squares) were treated with penclomedine at increasing concentrations for 24 h. (B) LN-18 puro (open circles) or LN-18 crm-A cells (closed circles) were incubated with different concentrations of penclomedine for 24 h. (C) LN-18 cells were treated with increasing concentrations of penclomedine in the absence (open squares) or presence (closed squares) of zVAD-fmk (200 μM), administered 2 h before penclomedine. Survival in panels (A–C) was measured by crystal violet staining. Data are expressed as mean percentages of survival and S.E.M. ($n = 3$). (D) LN-18 cells were treated with 0 (open squares), 60 (open circles) or 120 μM (open triangles) of penclomedine, or with CD95L (60 U/ml, closed circles). DEVD-amc-cleaving caspase activity was determined at the indicated time points. Data are expressed as mean fluorescence and S.E.M. ($n = 3$).

4. Discussion

The present study sought to examine the regulation of telomerase activity in human glioma cells and its significance for the response of cultured glioma cells to chemotherapy, specifically the putative telomerase inhibitor, penclomedine.

Telomerase activity was absent in untransformed astrocytes but was present in all 12 human glioma cell line (Fig. 1). The absence of telomerase in most human somatic ells and its presence in most (80–90%) human cancers has made telomerase activity an attractive parameter for staging, prognosis and therapy of cancer. Also, it has been suggested that telomerase may serve as an indicator of chemosensitivity or resistance since several different cytotoxic agents have been shown to inhibit telomerase activity in vitro (Burger et al., 1997; Pandita et al., 1997; Ishibashi and Lippard, 1998; Wang et al., 1998). Here, low telomerase activity was correlated with sensitivity to carmustine but not to various other cancer chemotherapy drugs (Table 1). This possible interrelation between carmustine sensitivity and telomerase activity may not be strong enough to suggest that telomerase activity might become a useful parameter to predict response to chemotherapy in human gliomas.

No induction of telomerase activity in response to bcl-2 gene transfer was observed here in glioma cell lines (Fig. 2A). Moreover, our observations regarding the lack of an association of p53 status and telomerase activity in glioma cell lines contrast with a correlation between telomerase activity and p53 protein accumulation in breast cancer and lung cancer in vivo (Roos et al., 1998; Wu et al., 1999) but are consistent with other studies where no such link became apparent in colorectal cancer in vivo and sarcoma cell lines in vitro (Brown et al., 1998; Milas et al., 1998). Moreover, disruption of p53 function in p53 knock-out mice did not affect telomerase activity in fibroblasts (Milas et al., 1998).

Interestingly, ectopic expression of mutant p53val^{135} (38.5°C) had no effect on telomerase activity in either cell line whereas ectopic express of wild-type p53val^{135} (32.5°C) led to a decrease of telomerase activity in p53 wild-type, but not mutant, cell lines (Fig. 2B). This observation is difficult to explain. The lack of suppression of telomerase activity in the p53 mutant cell lines cannot be attributed to a dominant negative effect of endogenous mutant p53 over the exogenous wild-type p53val^{135}, because LN-308 cells lack endogenous p53 altogether and because wild-type p53val^{135} induces growth arrest in all four cell lines, irrespective of endogenous p53 status (Trepel et al., 1998). Transduction of p53-negative human non-small-cell lung cancer cells with an adenoviral p53 vector led to a marked reduction in telomeric signals in FISH analysis, probably due to a loss of $(TTAGGG)_n$ repeats (Mukhopadhyay et al., 1998). Moreover, wild-type p53 H226b cells stably transfected with sense or antisense p53 cDNA showed a significant reduction or increase in telomerase activity. Since it has also been shown that p53 has exonucleolytic activity (Mummenbrauer et al., 1996), it is possible that high levels of p53 protein may directly degrade telomeric DNA and induce telomeric associations and subsequently apoptosis (Mukhopadhyay et al., 1998).

Penclomedine induced apoptotic, death receptor-, bcl-2- and caspase-independent cell death, but did not inhibit telomerase in glioma cells and did not act in synergy with other cytotoxic drugs (Figs. 5–7). While the cell death pathway mediating penclomedine cytotoxicity in glioma cells remains obscure at a biochemical level, this cell death is an interesting paradigm of presumably caspase-independent death in tumor cells.

In summary, we conclude that telomerase activity is not a suitable target to enhance the chemosensitivity of human malignant gliomas and that telomerase activity in these cells is bcl-2-independent but subject to complex regulation by p53.

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