Ann Surg Oncol
DOI 10.1245/s10434-011-1644-0

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Butylidenephthalide Suppresses Human Telomerase Reverse Transcriptase (TERT) in Human Glioblastomas

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ABSTRACT

Background. Telomerase is widely expressed in most human cancers, but is almost undetectable in normal somatic cells and is therefore a potential drug target. Using the human telomerase promoter platform, the naturally occurring compound butylidenephthalide (BP) was selected for subsequent investigation of antitumor activity in vitro and in vivo.

Methods. We treated human glioblastoma cells with BP and found a dose-dependent decrease in human telomerase reverse transcriptase (*hTERT*) mRNA expression and a concomitant increase in p16 and p21 expression. Because c-Myc and Sp1 are involved in transcriptional regulation of *hTERT*, the effect of BP on c-Myc and Sp1 expression was examined.

Results. Using electrophoretic mobility shift assays and western blotting, we showed that BP represses *hTERT* transcriptional activity via downregulation of Sp1 expression. Using the telomerase repeat amplification protocol, an association between BP concentration and suppression of telomerase activity, induction of human glioblastoma senescence, and inhibition of cellular proliferation was identified. This was supported by a mouse xenograft model, in which BP repressed telomerase and inhibited tumor proliferation, resulting in tumor senescence. Overexpression of

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Electronic supplementary material The online version of this article (doi:10.1245/s10434-011-1644-0) contains supplementary material, which is available to authorized users.

First Received: 6 May 2010

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Published online: 07 May 2011

hTERT restored telomerase activity in human glioblastoma cells and overcame replicative senescence.

Conclusions. These findings suggest that BP inhibits proliferation and induces senescence in human glioblastomas by downregulating *hTERT* expression and consequently telomerase activity. This is the first study to describe regulation of telomerase activity by BP in human glioblastomas.

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults. The current standard-of-care therapies include surgery, radiation, and adjuvant temozolomide (TMZ) treatment.[1] Although TMZ prolongs survival for GBM patients, the degree of benefit is modest, and the effect is restricted to GBM patients with methylated O^6-methylguanine-DNA methyltransferase.[2] Therefore, development of new targeted drugs for GBM therapy is needed.

The telomerase enzyme uses an RNA template to synthesize and maintain telomere sequences by the addition of terminal TTAGGG sequences. Telomerase is pertinent to a cell's potential for self-renewal and is required for long-term cellular proliferation and survival.[3,4] Because human cells progressively lose telomerase activity, telomere DNA is lost during successive rounds of DNA replication. Shortening of telomeres has been reported to correlate with cellular senescence.[5,6] This indicates that replicating cells that lose the telomerase needed to maintain telomeres must eventually senesce.[7]

Telomerase is composed of 2 major subunits. The human telomerase RNA component (*hTR*) subunit is an RNA that serves as a template for synthesis of the telomere repeats and is essential for telomerase activity.[8] The other subunit is a catalytic subunit called human telomerase reverse transcriptase (hTERT).[9] In Counter's studies, transient transfection of an hTERT expression plasmid has been demonstrated to restore telomerase activity to noncancerous human cells that lack hTERT and telomerase activity but express *hTR*, implicating hTERT as the rate-limiting step for telomerase activity.[10]

In humans, telomerase is expressed in adult germ-line tissues, in stem cells, and during embryonic development, but is absent in most human somatic tissues.[7,11] Unlike in normal somatic cells, 80%–90% of human tumors exhibit telomerase activity. Telomeres in tumor cells are relatively short, but are maintained at a certain length, probably as a result of telomerase function. On the other hand, tumors lacking telomerase activity maintain telomeres by a recombination-mediated process termed alternative lengthening of telomeres.[12] In most cancers, the level of telomerase activity generally correlates with the proliferation state of the cells. The presence of the enzyme is required for unlimited proliferation (immortality), whereas its absence almost always dictates a finite lifespan (senescence).[13] This suggests that telomerase activation is involved in establishment of cellular immortality and may therefore be a critical step in carcinogenesis.[10]

In human brain tissues, telomerase activity is observed in 89% of glioblastomas and 45% of anaplastic astrocytomas, but is absent in normal brain tissues.[14] Therefore, development of a telomerase inhibitor may provide a therapeutic strategy for selective targeting of malignant gliomas, sparing normal brain tissue.[15]

In our previous study, we screened traditional Chinese medicines (TCM) for new telomerase inhibitors using a telomerase promoter/luciferase reporter system.[16] Using this system, we identified the chloroform extract of *Angelica sinensis* (AS-C) as a drug candidate that decreases telomerase promoter activity. *Angelica sinensis* (also known as dong quai) is indicated for menstrual disorders, including menopausal symptoms.[17] It has also been widely used for conditions such as gastric mucosal damage, hepatic injury, impaired myocardial blood flow, and chronic glomerulonephritis.[18–21] Furthermore, dong quai has been promoted in the United States for treatment of several gynecologic disorders.[22]

AS-C has also exhibited antitumor activity by causing growth arrest and apoptosis of glioblastomas both in vitro and in vivo.[23] Subsequently, we separated the major compounds from AS-C and identified butylidenephthalide (BP) as a major component (more than 30% of crude AS-C).[24] BP has a dramatic antitumor effect, causing G0/G1 growth arrest and apoptosis of glioblastoma and hepatocellular carcinomas both in vitro and in vivo.[24–26] In vitro, BP promotes both p53-dependent and independent apoptosis. In vivo, BP suppresses tumor growth and reduces the volume of GBM in situ. These findings suggest that BP is a potential anticancer compound for clinical treatment of GBM.

In the present study, we report that the naturally occurring compound BP is a telomerase suppresser and demonstrate the antitumor mechanism of BP in human brain cancer cells. Using 2 human glioblastoma brain cancer cell lines, DBTRG-05MG and 8401, we show that BP suppresses cellular proliferation and inhibits telomerase activity by repressing *hTERT* transcriptional activity. Moreover, upon BP treatment, brain tumor cells became senescent concomitant with suppression of telomerase activity. Taken together, these data suggest that the antiproliferative and senescence effects of BP result from inhibition of telomerase activity, providing new insight into the effects of BP on brain cancers.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human GBM cell lines DBTRG-05MG (BCRC 60380) and GBM 8401 (BCRC 60163) were obtained from

Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). The cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Butylidenephthalide (BP; Lancaster Synthesis Ltd., Newgate, Morecambe, UK) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mg/mL and stored at −20°C. Carmustine (BCNU), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, β-actin, and FITC-conjugated secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium, FBS, penicillin, streptomycin, and trypsin/EDTA were purchased from Biowest (Nuaille, France). The Annexin V-FLOUS Staining Kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Telomerase antibody was purchased from Abcam (Cambridge, UK). Polyvinylidene fluoride (PVDF) membranes, BSA protein assay kits, and Western Lightning Chemiluminescence Reagent Plus were purchased from Amersham Biosciences (Arlington Heights, IL).

MTT Assay

Viable cells were evaluated using a modified MTT assay.[24] Briefly, cells were plated in a 96-well plate. The experiment was performed in a 96-well plate. Toward the end of the experiment, the medium was replaced with 100 μL fresh medium containing MTT (500 μg/mL) and cells were incubated for 4 h at 37°C. The MTT medium was removed, and 100 μL DMSO was added to each well. Absorbance was measured using a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT) at 570 nm. Absorbance of untreated cells was designated as 100%. Data represent the mean ± SD from three independent experiments.

Reverse Transcriptase PCR (RT-PCR)

Cells were treated with 70 μg/mL BP for 0.5, 1, 3, or 6 h. As a treatment control, vehicle (DMSO) was used. After treatment, cells were washed with phosphate-buffered saline (PBS), pH 7.4, and removed from plates using trypsin. Total RNA from each sample was isolated using the RNeasy Midi Kit and the RNase-free DNase Set (Qiagen, Valencia, CA). Total RNA concentration was determined using UV spectrophotometry and adjusted to 1 μg/μL. RNA quality was examined using 1.5% agarose gel electrophoresis with ethidium bromide staining. To generate cDNA, total RNA (1 μg) was reverse-transcribed using the Omniscript RT kit (Qiagen), and the cDNA (1 μg) was amplified by PCR in the presence of 1 μmol primers and *Taq* DNA polymerase (Takara Shuzo Company, Shiga, Japan): 95°C for 10 min; 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Amplification products were analyzed using a GS-800 Calibrated Imaging Densitometer (Quantity One 4.0.3 software; Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Cells were cultured in 100-mm^2 dishes and incubated in various concentrations of BP for the indicated times. The cells were lysed on ice in 200 μL lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $MgCl_2$, 0.5 vol% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 50 μg/mL leupeptin) and centrifuged at 13,000×g for 20 min at 4°C. Soluble protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories). Lysate supernatants (50 μg) were subjected to SDS-PAGE analysis. Resolved proteins were transferred to PVDF membranes, blocked overnight in 5% nonfat milk, and probed with an appropriate dilution of primary antibody for 1 h at room temperature. Membranes were then washed 3 times with PBS containing 0.1 vol% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western Lightning Chemiluminescence Reagent Plus and quantified by densitometry.

Electrophoretic Mobility Shift Assay (EMSA)

DBTRG-05MG cells were plated in 10-cm dishes and allowed to reach 80% confluency. Cells were treated without (−) or with (+) BP (70 μg/mL) for 48 h. DBTRG-05MG cell nuclear extracts were prepared and subjected to electrophoretic mobility shift assays using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). The binding reactions contained 2 μg nuclear extract, binding buffer (final 10 mM Tris pH 7.5, 50 mM KCl, 5 mM $MgCl_{1,\ 2}$ mM dithiothreitol, 0.05 vol% Nonidet P-40, 2.5 vol% glycerol), 1 μg poly(dI-dC), and 2 μM biotin end-labeled oligonucleotide containing a putative binding site for Sp1 (Table 1). Binding reactions were incubated at 25°C for 20 min, and DNA-protein complexes were subjected to 6% native polyacrylamide gel electrophoresis in 0.5 × Tris borate/EDTA (TBE) buffer at 100 V for 3 h and transferred onto Hybond-N^+ nylon membrane (Millipore, Billerica, MA) in the same buffer at 100 V for 1 h. The membrane was immediately cross-linked for 5 min under a UV transilluminator equipped with 312-nm bulbs, and complexes containing biotin-labeled probe were detected using chemiluminescence.

Telomerase Activity Assays

Telomerase activity was assessed using the telomerase repeat amplification protocol (TRAP) assay. DBTRG-05MG cells were plated in 6-well plates (1 × 10^5 cells/well). After

TABLE 1 Gene-specific RT-PCR primer sequences and EMSA probe sequences

Oligonucleotide sequence	Product size (bp)
RT-PCR Primer	
hTERT1	145
F; 5′-CGGAAGAGTGTCTGGAGCAA-3′	
R; 5′-GGATGAAGCGGAGTCTGGA-3′	
hTERT2	457
F; 5′-GCCTGAGCTGTACTTTGTCAA-3′	
R; 5′-CGCAAACAGCTTGTTCTCCATGTC-3′	
hTR	103
F; 5′-CTAACCCTAACTGAGAAGGGCGTAG-3′	
R; 5′-GAAGGCGGCAGGCCGAGGCTTTTCC-3′	
p21	350
F; 5′- CCTCTTCGGCCCGGTGGAC -3′	
R; 5′- CCGTTTTCGACCCTGAGAG -3′	
$p16^{INK4a}$	409
F; 5′-CCCAACGCACCGAATAGTTAC-3′	
R; 5′-CACGGGTCGGGTGAGAGT-3′	
GAPDH	965
F; 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	
R; 5′-CATGTGGGCCATGAGGTCCACCAC-3′	
EMSA Probe	
Sp1	N/A
5′-ATTCGATCGGGGCGGGGCGAG-3′	

F forward, *R* reverse, *N/A* not applicable

24 h, cells were incubated with the indicated concentration of BP (0–100 μg/mL) for 3 days and chased with medium lacking BP for another 3 days. Total cell extracts (3 μg) were subjected to the TRAP ELISA assay.[27] Cell extracts from immortalized telomerase-expressing human kidney cells (293 cells) were used as a positive control. The absorbance of samples were measured using a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT) at 450 nm. Telomeric DNA products were separated by 8% nondenaturing PAGE in 0.5 × Tris-borate-EDTA buffer (pH 8.3) at 100 V for 1 h. Gels were then stained with SYBR Green I, and image was captured with BioDoc-It Imaging System (Upland, CA). Reaction products were examined for ribonuclease sensitivity to ensure that they resulted from telomerase activity. Telomerase activity was assigned arbitrary units, with all activities normalized to that from control DBTRG-05MG cells (no BP). Data represent the mean ± SD from 3 independent experiments.

β-Galactosidase Histochemical Staining

To determine β-galactosidase expression in DBTRG-05MG cells, 1×10^4 DBTRG-05MG cells were plated in a Nunc 8-well Chamber Slide (Apogent Technologies, Portsmouth, NH) and incubated for 48 h. Cells were treated with various concentrations of BP (0–100 μg/mL) for 6 days, during which medium containing BP was replaced every 3 days. The Senescence β-Gal Staining Kit (United States Biological, Swampscott, MA) was used to detect senescent cells. Cells were washed twice with PBS and fixed with a 2 vol% formaldehyde solution containing 0.2 vol% glutaraldehyde for 5 min. The cells were then washed with PBS and stained using 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; diluted from a 20 mg/mL stock in dimethylformamide), in 40 mM citric acid and sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$ as described (United States Biological, Swampscott, MA). Following overnight incubation at 37°C, the cells were washed twice with PBS and the percentage of positively stained cells was determined using a light microscope (400×) and images obtained. For quantification of SA-β-Gal positive cells we follow Kurz et al.'s report.[28] The percentage of SA-β-Gal positive cells was determined by counting the number of blue cells under bright field illumination and then the total number of cells in the same field under phase contrast.

In vitro Transfection

To create a stable TERT-expressing 8401 cell line, 8401 cells were transfected with 2 μg pCI-neo-hEST2-HA (phTERT) plasmid 1782 (Addgene, Inc., Cambridge, MA) or pCI neo vector (pCI) (Promega, Madison, WI) using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA).[10] After 48 h, the cells were subjected to selection for stable integrants by exposure for 3 weeks to 500 μg/mL G418 (Invitrogen) in complete medium containing 10% FBS. The cells were then assessed for overexpression of TERT by Western blot analysis.

Colony Formation Assay

A total of 8401 cells stably transfected with phTERT or pCI were plated onto 6-well culture dishes at 100 cells/well in 2 mL of culture medium in the presence of BP for 24 h. BP was removed and the cells were cultured in fresh medium for 10–15 days. When colonies became visible, they were stained with 2% methyl blue solution to assess the survival rate. Single colonies were selected and expanded separately. The data represent mean ± SD from 3 independent experiments.

Flow Cytometric Determination of Cell-Cycle Distribution Using Propidium Iodide

To determine the phase distribution of cellular DNA, propidium iodide (PI) staining was performed. Briefly,

2×10^6 cells were collected and washed twice with ice-cold PBS and fixed overnight in 70% ethanol. After centrifugation at $400\times g$ for 5 min at 4°C, cell pellets were treated with extraction buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.8) for 10 min at room temperature in the dark, and pelleted again by centrifugation at $400\times g$ for 5 min at 4°C. Cell pellets were stained with PBS containing 5 μg/mL propidium iodide (PI; Sigma), 0.5 mg/mL RNaseA (Sigma), and 1% Triton X-100 for 30 min at room temperature in the dark. Cell-cycle analysis was performed using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA). Cell-cycle distributions were analyzed using Cell-Quest and Modfit software (Becton-Dickinson).

Animal Studies

Tumorigenesis assays were performed using a xenograft mouse model as described previously.[24] The human GBM cell line GFP-DBTRG-05MG, stably transfected with various GFP-tagged constructs, was a gift from Tzyy-Wen Chiou (National Dong Hwa University, Taiwan). GFP-DBTRG-05MG cells (2×10^6) were injected subcutaneously (sc) into the backs of nude mice. The volume of each tumor was measured twice a week after tumors were detected by an IVIS imaging system (Xenogen, Alameda, CA). Tumors were measured with calipers, and the volume was calculated using the equation $L \times H \times W \times 0.5236$.[29] When tumors reached ~ 50 mm^3, mice were grouped ($n = 3$ per group) and then treated for 5 days with vehicle or with 100, 300, or 500 mg/kg/day BP (injected sc in the back, opposite to the tumor). At 30 days after treatment, mice were sacrificed, and tumor samples were examined by Western blotting and immunohistochemical staining.

Immunohistochemical Staining

Xenograft mice models were established and have been described previously.[24] Tumor tissues (subcutaneous DBTRG-05MG tumors with or without BP treatment) were harvested and fixed in 4% neutral buffered formalin. Paraffin-embedded sections were prepared from the tumors and processed for immunohistochemical staining. Sections were incubated overnight in rabbit polyclonal anti-telomerase or anti-Ki67 antibody (1:100 dilutions; Abcam, Cambridge, UK) at 4°C. The immune complexes were visualized using horseradish peroxidase-conjugated anti-goat IgG secondary antibody (1:1000 dilution; Santa Cruz Biotechnology, San Diego, CA), with amplification using the LSAB2 system (DAKO, Corp., Carpinteria, CA) and detection by incubation for 10 min in PBS containing 0.5 mg/mL diaminobenzidine and 0.03 vol% H_2O_2. Finally, sections were counterstained with hematoxylin and mounted. To determine senescence-associated β-gal activity, tissues were snap frozen in OCT mounting medium (Gene Research Lab. CO. Ltd., Taipei, Taiwan), and 12-μm sections were stained. Cells were observed under a light microscope (400×), and images were obtained.

Statistical Analysis

The data represent mean ± SD. Statistical differences were analyzed using the t test for normally distributed values and the nonparametric Mann-Whitney U test for values of non-normal distribution. For the pairwise comparisons of multiple samples, statistical differences were analyzed using the t test to compare the specific pairs of groups in 1-way ANOVA (LSD procedure) for normally distributed values and to compare the specific pairs of groups under the Kruskal-Wallis test (Dunn procedure) values of non-normal distribution. Values of $P < .05$ were considered significant.

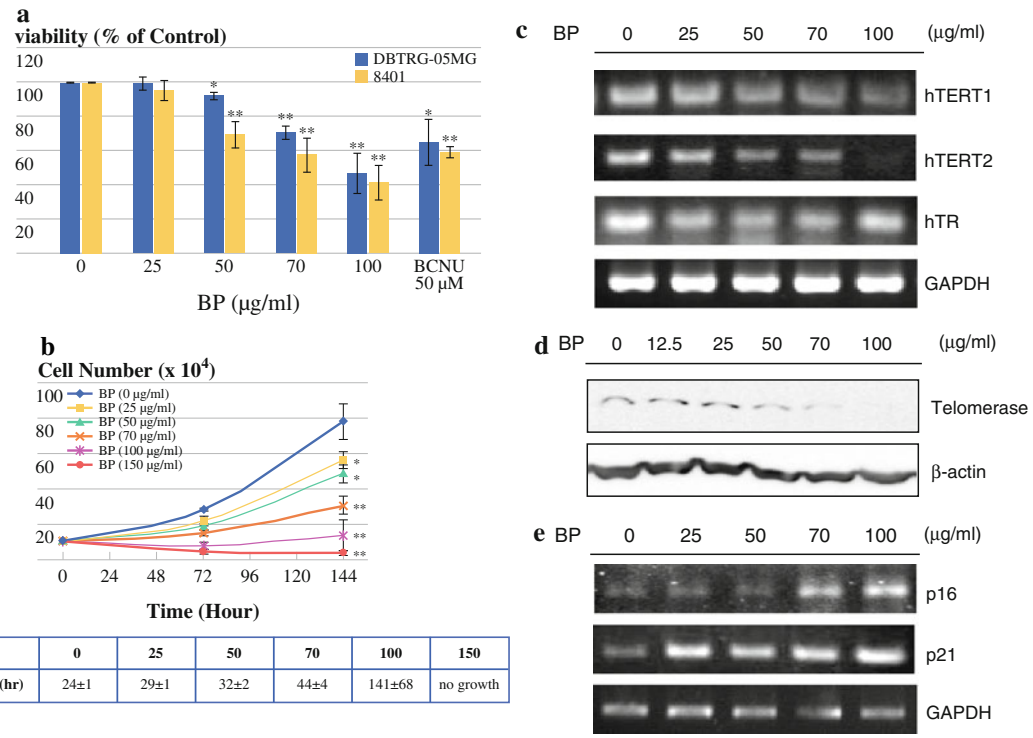
RESULTS

BP Inhibits Growth of Human Brain Cancer Cells

Exposure of human glioblastoma cells (DBTRG-05MG and 8401 cells) to BP (25–100 μg/mL) resulted in a dose-dependent decrease in cell viability (Fig. 1a). Because cell viability is regulated by apoptotic and proliferative, we counted cell number to calculate the cell's doubling time. The DBTRG-05MG cell's doubling time is around 24 h. After BP treatment, we found the cells doubling time extension according to BP concentration (Fig. 1b). No cell number increase was detected during treatment with 150 μg/mL of BP. These results suggest that BP inhibits growth of human brain cancer cells that are dose dependent.

BP Downregulates hTERT Expression and Upregulates p16 and p21 Expression

Because many cancer cells have high telomerase activity, which results in increased survival and proliferation, we examined whether BP represses telomerase activity in brain cancer cells. Following the Ulaner's report, PCR primer hTERT1 amplifies a 145-base region near the 5′ end of the reverse transcriptase domain.[7] PCR primer hTERT2 amplifies a region of the *hTERT* mRNA within the reverse transcriptase domain. Primer hTERT1 was designed for detecting the *hTERT* transcription level. Primer hTERT2 was designed for *hTERT* to see whether posttranscription alternate splicing would occur. We observed that BP treatment (25–100 μg/mL) of DBTRG-05MG cells for 48 h decreased *hTERT* mRNA expression (Fig. 1c) in a dose-



BP (μg/ml)	0	25	50	70	100	150
Doubling time (hr)	24±1	29±1	32±2	44±4	141±68	no growth

FIG. 1 BP inhibits brain tumor cell growth and suppresses telomerase expression. **a** DBTRG-05MG and GBM 8401 cells were treated with 0–100 μg/mL BP for 48 h. Cell viability was determined by MTT assay and expressed as a percent relative to untreated cells. **b** 1×10^5 DBTRG-05MG cells were cultured for 24 h in 6-well plates and then incubated with the indicated BP concentration for 72 h followed by incubation in BP-free medium for an additional 72 h. At the indicated times, viable cells were counted using trypan blue exclusion. The cell doubling time is shown. **c** RT-PCR analysis of telomerase mRNA expression following BP treatment. DBTRG-05MG cells were plated in 6-well plates and allowed to reach 60% confluence. Cells were then treated with the indicated BP concentration for 48 h. Primer hTERT1 was designed for detecting the *hTERT* transcription level. Primer hTERT2 was designed for detecting *hTERT* to see whether posttranscription alternate splicing would occur. The expression of hTERT1, hTERT2, and hTR transcripts was analyzed by RT-PCR using primers specific for each gene. Amplification products were subjected to 1% agarose gel electrophoresis. GAPDH mRNA was analyzed as a loading control. **d** Western blotting analysis of telomerase expression following BP treatment. DBTRG-05MG cells (1×10^5) were plated in 6-well plates and allowed to reach 60% confluence. Cells were then treated with the indicated BP concentration for 48 h and subjected to Western blot analysis using anti-telomerase. *β*-actin was analyzed as a loading control. **e** The expression of *p16* and *p21* transcripts was analyzed by RT-PCR using primers that are specific for each gene. Amplification products were subjected to 1% agarose gel electrophoresis. *GAPDH* mRNA was analyzed as a loading control. $*P < .05$, $**P < 0.01$

dependent manner. However, the expression of *hTR* RNA (an RNA template for synthesis of the telomere repeats) did not change (Fig. 1c). Moreover, the expression of telomerase protein decreased in a dose-dependent manner (Fig. 1d). To investigate whether the repression of telomerase activity by BP was related to brain cancer cell proliferation, *p16* and *p21* mRNA expression were examined. As a result, *p16* and *p21* mRNA expression in DBTRG-05MG cells increased (Fig. 1e) following treatment with the same concentrations of BP that resulted in decreased *hTERT* expression (Fig. 1c).

BP Downregulates Sp1 Expression in Brain Cancer Cells

Because Sp1 binding sites are located in the *hTERT* core promoter region (−180 bp), we were interested in investigating whether BP alters Sp1 expression in DBTRG-05MG cells brain cancer cells. Using EMSA analysis of nuclear extracts, we observed that BP treatment decreased Sp1 binding site complex formation (Fig. 2a). Similarly, Sp1 expression also temporally decreased following treatment of DBTRG-05MG cells with 70 μg/mL BP (Fig. 2b). These results showed that BP downregulates Sp1 expression, which could be a potential mechanism by which telomerase activity is repressed in brain cancer cells by BP.

BP Represses Telomerase Activity in Brain Cancer Cells

Expression of *hTERT* is tightly regulated in association with telomerase activity in various cancer cells.[30–33] Using the TRAP assay, we determined that BP repressed telomerase activity in DBTRG-05MG cells in a dose-

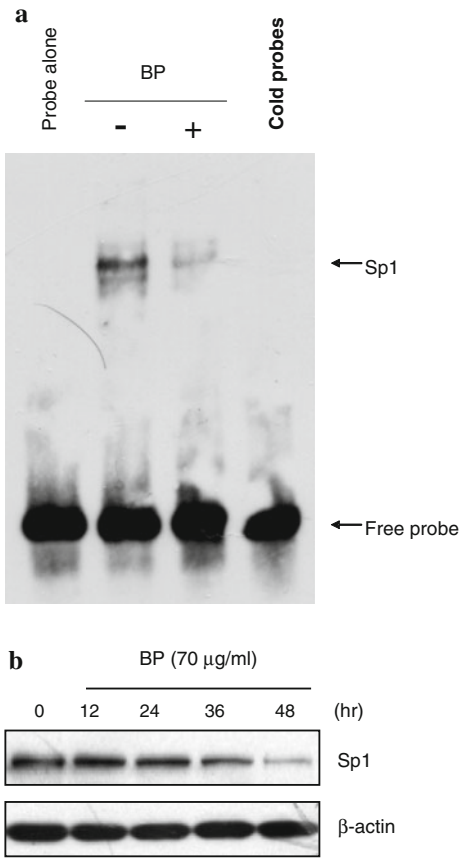


FIG. 2 BP represses *hTERT* transcription via downregulation of Sp1 expression. **a** DBTRG-05MG cells were plated in 10-cm dishes and allowed to reach 80% confluency. Cells were treated without (−) or with (+) BP (70 μg/mL) for 48 h. Cell nuclear extracts were prepared and subjected to chemiluminescent EMSA. Reactions contained 2 μg nuclear extract and 2 μM biotinylated oligonucleotide probes containing Sp1 binding site. Probe alone: Labeled Sp1 probes only with no sample. Cold probes: BP treated sample with cold and labeled Sp1 probes. **b** DBTRG-05MG cells (80% confluent) were treated without or with BP (70 μg/mL) for 0, 12, 24, 36, and 48 h. Whole-cell extracts were prepared at the indicated time and subjected to Western blot analysis using anti-Sp1 antibody. *β*-actin was analyzed as a loading control

dependent manner (Fig. 3a and b). This suggests that the observed BP-dependent downregulation of telomerase expression in DBTRG-05MG cells results in concomitant repression of telomerase activity.

BP Induces Senescence in Brain Cancer Cells

It has been suggested that replicating cells that lack telomerase to maintain telomeres must eventually senesce.[7] To evaluate the effect of BP on DBTRG-05MG cell senescence, we used the senescence-specific stain senescence-associated *β*-galactosidase (SA-*β*-gal). In contrast to untreated cells, staining of BP-treated DBTRG-05MG cells was considerably increased. The majority of cells treated with BP displayed light to intense staining depending on BP concentration (Fig. 3c and d). The adherent cells that remained following BP treatment were enlarged and flattened and exhibited a decreased saturation density, indicating entry into a state resembling senescence. Thus, the appearance of a senescence-like state in DBTRG-05MG cells was closely associated with BP suppression of telomerase activity.

Exogenous hTERT Expression Rescues Telomerase Activity and Decreases Senescence in Brain Tumor Cells Treated with BP

To investigate the role of telomerase in BP-induced senescence in brain tumor cells, cell lysates from GBM 8401 cells transiently transfected with either pCI (empty vector) or phTERT (hTERT expression) were subjected to SDS-PAGE analysis followed by Western blot analysis. Immunoblotting using HA antibody indicated that a ∼ 130-kDa exogenous hTERT polypeptide (arrow, hTERT-HA) was detected in the phTERT clone but not in pCI clone (Fig. 4a, right). In addition, telomerase activity in these cell lines was analyzed using the TRAP assay. BP treatment decreased telomerase activity in the pCI clone in a dose-dependent manner (Fig. 4b, left), but no significant decrease was observed in the phTERT clone (Fig 4b, right). Moreover, SA-*β*-gal staining was reduced in the phTERT clone (Fig. 4c, upper right) compared with the pCI clone following BP treatment. These results indicate that BP-mediated suppression of telomerase activity and induction of senescence was negated by exogenous hTERT expression.

Antiproliferation and Senescence Effects of BP are Linked to Inhibition of Telomerase Activity

To examine the effect of BP treatment on cell proliferation, the GBM 8401 phTERT clone was treated with BP and subjected to immunofluorescence staining using the proliferation-specific antibody against Ki67. After BP treatment, Ki67 expression was suppressed in the pCI clone, but persisted in the phTERT clone (Fig. 5a). Flow cytometry analysis indicated that treatment with 70 μg/mL BP resulted in cell-cycle arrest at the G0/G1 phase in the pCI clone (Fig. 5b). In addition, BP also decreased the percentage of pCI clones that entered the G2/M phase. Compared with pCI clone, the phTERT clone did not exhibit a change in cell-cycle arrest after BP treatment (Fig. 5b). This suggests that antiproliferation and cell-cycle arrest effects of BP are associated with telomerase activity.

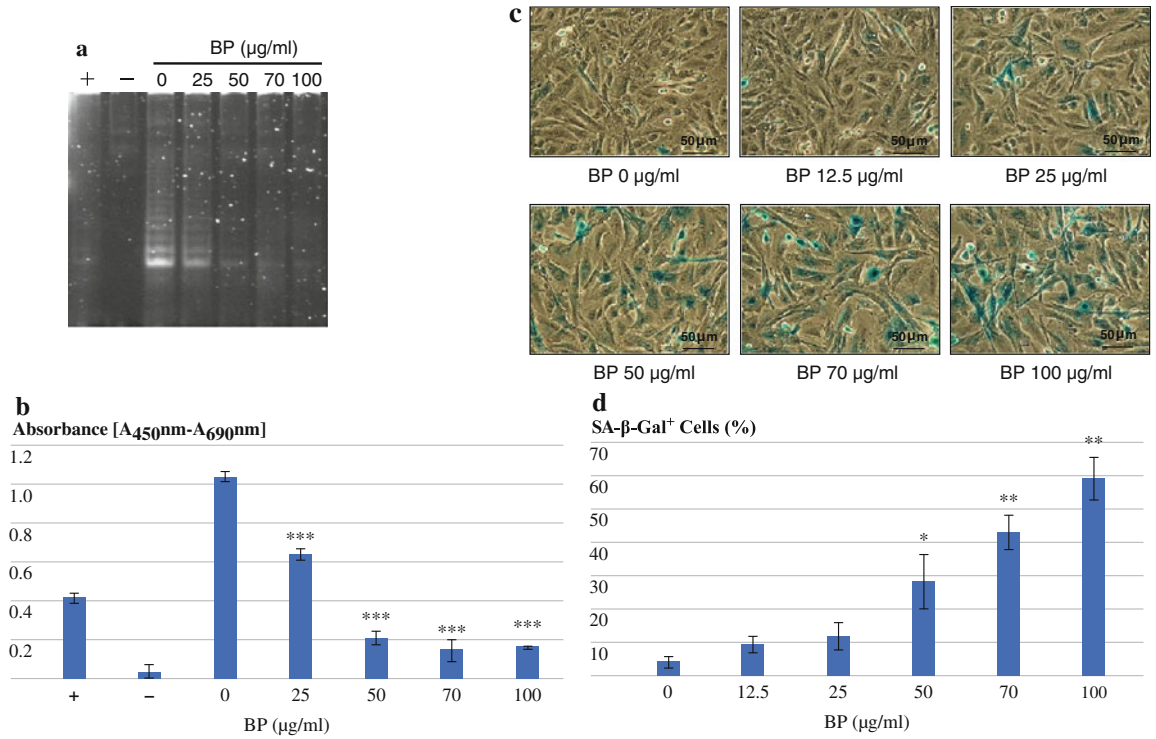


FIG. 3 BP suppresses telomerase activity and induces senescence in glioblastoma cells. **a** DBTRG-05MG cells were plated in 6-well plates (1×10^5 cells/well). After 24 h, cells were incubated with the indicated concentration of BP for 3 days followed by incubation in BP-free medium for another 3 days. Total cell extracts (3 μg) were subjected to TRAP ELISA analysis. Cell extracts from telomerase-expressing 293 cells (+) or from heat treated 293 cell (−) were used as controls. **b** Quantitative estimations of telomerase activity. The absorbance of samples were measured using a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT) at 450 nm (right). ***$P < .001$. **c** DBTRG-05MG cells (1×10^4) were plated in 8-well Chamber Slides (Nunc, Apogent Technologies, Portsmouth, NH) and incubated for 48 h. Cells were treated with the indicated concentration of BP for 6 days, and BP was replaced every 3 days. Senescent cells (*green*) were detected using the Senescence β-Gal Staining Kit (United States Biological, Swampscott, MA), and representative images were obtained under light microscopy (bar = 50 μm). **d** Quantification of SA-β-Gal positive cells. The results represent the average of the 3 independent experiments. *$P < .05$, **$P < .01$

BP Inhibition of Human Glioblastoma Cell Growth in a Xenograft Nude Mouse Model is Associated with Decreased hTERT Expression

To evaluate the antitumor activity of BP in vivo, human brain cancer xenografts were established by subcutaneous injection of GFP-DBTRG-05MG cells ($\sim 2 \times 10^6$) into the dorsal subcutaneous tissue of nude mice. After tumors reached approximately 50 mm^3, mice were randomized into control and treatment groups ($n = 3$) and given a daily subcutaneous injection of vehicle alone (control group) or 100, 300, 500 mg/kg of BP (treatment groups) for 5 successive days. In the following days observed, the tumors were found to be effectively inhibited, which differed from those treated with the control with vehicle only (Fig. 6a and c). In addition, the body weight did not change in the observed days. Western blot analysis of tumor lysates indicated that telomerase protein was decreased in the tumor tissues following BP treatment (Fig. 6b). Immunohistochemical analysis showed that downregulation of telomerase and Ki67 expression in the tumor tissues correlated with BP concentration (Fig. 6e, upper and middle panels). In addition, SA-β-gal staining became intense, indicating that the human glioblastomas became senescent in association with BP concentration (Fig. 6e, lower panels).

DISCUSSION

Current cancer therapies have limited efficacy for brain cancer patients, and new therapeutic agents are therefore needed. Unlike chemotherapeutic agents, which elicit systemic effects, targeted drugs inhibit cancer cell growth while having only minimal effect on healthy cells. In general, although telomerase activity is absent in normal brain tissues, 80%–90% of human tumors have active telomerase for telomere maintenance. Importantly, 89% of glioblastomas and 45% of anaplastic astrocytomas exhibit telomerase activity.[14] Therefore, telomerase may be a suitable target for selective treatment of malignant gliomas.[15]

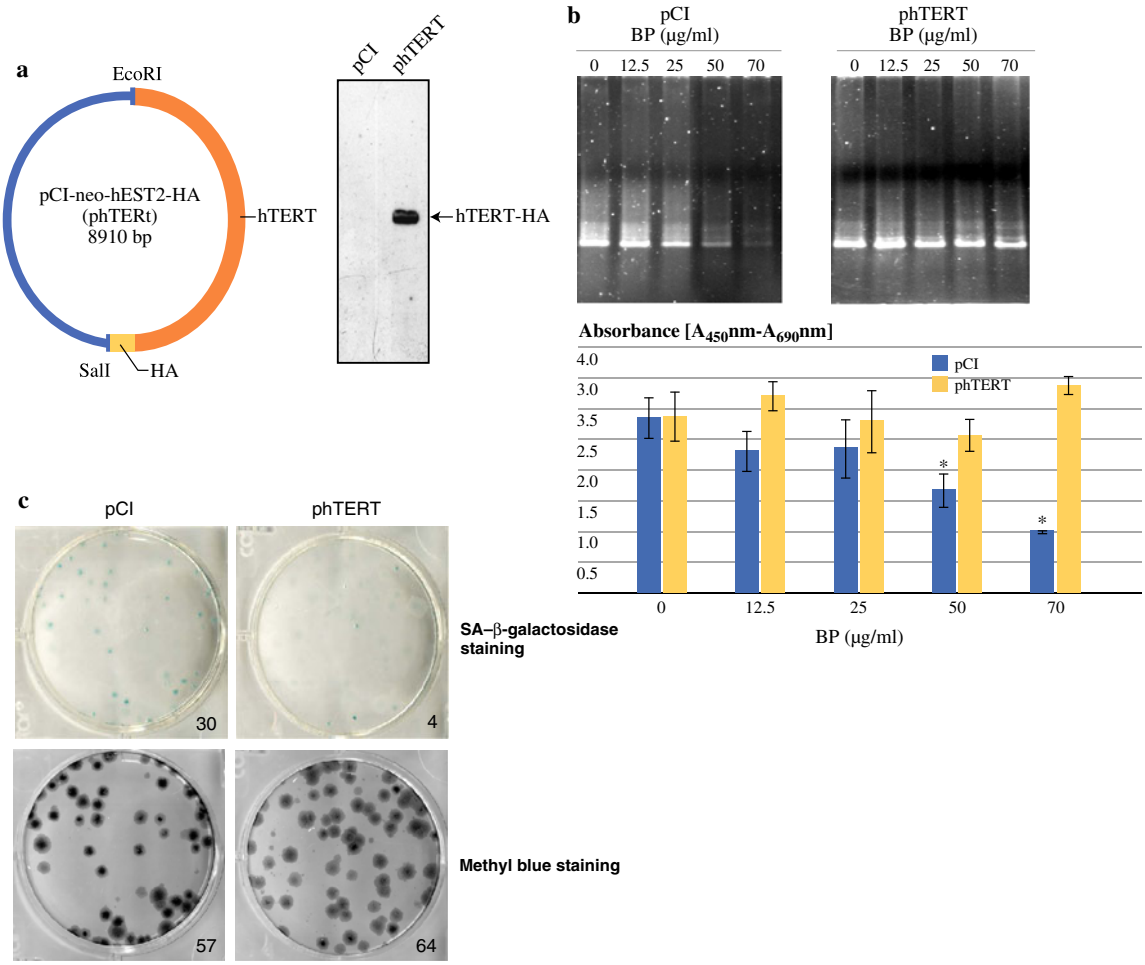


FIG. 4 Exogenous hTERT expression rescues telomerase activity and decreases senescence in brain tumor cells treated with BP. **a** *Left panel*: Map of the phTERT vector. Right panel: GBM 8401 cells were stably transfected with either pCI-neo vector (pCI) or pCI-neo-hEST2-HA (phTERT), and cell lysate supernatants were subjected to immunoblot analysis using a HA mouse mAb (1:1000, Cell Signaling). A ~130 kDa of exogenous hTERT protein was detected in GBM 8401 phTERT clones but not in pCI clones. **b** GBM 8401 pCI or phTERT clones were incubated with the indicated amount of BP for 3 days followed by incubation in BP-free medium for another 3 days. Total cell extracts (3 μg) were subjected to TRAP ELISA analysis (upper panels). The absorbance of samples were measured using a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT, USA) at 450 nm (lower panel). $*P < .01$. **c** GBM 8401 pCI or phTERT clones were incubated with 70 μg/mL of BP for 24 h and then grown in culture medium for 2 weeks and examined for colony formation. Senescent cells (*green*) were stained using the Senescence *β*-Gal Staining Kit and surviving cells were stained using methyl blue. The numbers in the lower right corner of each image are the cell number of positive stain

Many compounds specifically targeted against telomerase have recently been developed and tested. These compounds fall into several broad categories depending on whether they target the catalytic subunit of telomerase (hTERT), the RNA template subunit of telomerase, or G-quartet structures.[34] For example, HDAC inhibitors such as trichostatin A (TSA) and sodium butyrate (NaB) downregulate telomerase activity in prostate cancer cells by suppressing *hTERT* mRNA expression.[35] GRN163L, an oligonucleotide complementary to *hTR*, was reported to inhibit telomerase activity in human cancer cell lines derived from hepatomas, breast cancers, and glioblastomas.[15,36,37] BRACO19 and RHPS4 are G-quadruplex-stabilizing agents.[38,39] Many reports suggest that the expression of hTERT is tightly correlated with telomerase activity.

To screen for novel telomerase suppressors as antitumor target drugs, we previously constructed *hTERT* promoter/luciferase reporter system as a drug screening platform. *Angelica sinensis*, a Chinese herbal medicine, was identified as a candidate for future study. In addition, butylidenephthalide (BP), which was isolated from the chloroform extract of *Angelica sinensis,* demonstrated a dramatic antitumor effect both in vitro and in vivo.[24–26] In the present study, we investigated the molecular mechanisms underlying the anticancer effects of BP in brain cancer cells.

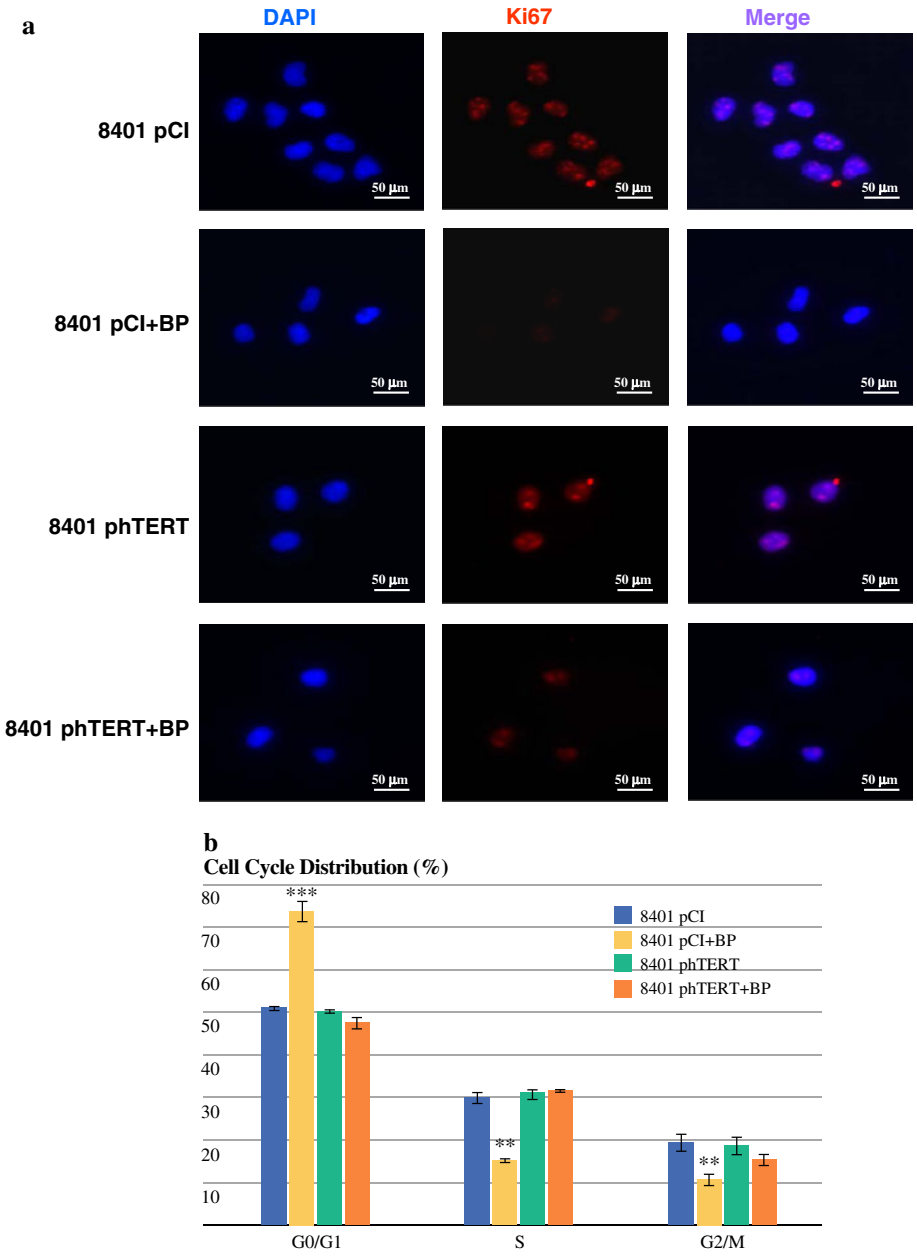


FIG. 5 Stable expression of hTERT overcomes BP antiproliferation effect in brain tumor cells. **a** GBM 8401 pCI or phTERT clones were plated on chamber slides and treated without or with 70 μg/mL BP for 24 h. Cells were then fixed, immunostained (*red*) using antibody against Ki67, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; *blue*). The fluorescent images were visualized with a fluorescence microscope (bar = 50 μm). **b** Cell-cycle analysis of GBM 8401 pCI or phTERT clones. Cells were cultured with (+) or without (−) 70 μg/mL BP for 24 h. The percentage of cells in the indicated cycle phase was determined using FACS analysis following propidium iodide (PI) staining. The results represent the average of the 3 independent experiments. $^{**}P < .01$, $^{***}P < .001$

We first examined the effect of BP on transcriptional regulation of *hTERT* and revealed that BP elicits a dose-dependent decrease in hTERT mRNA and protein expression, but does not affect *hTR* RNA expression. Decreased hTERT expression resulted from transcriptional inhibition of *hTERT* and was also associated with decreased telomerase activity (Fig. 3a and b). Using 2 brain cancer cell lines, DBTRG-05MG and GBM 8401, we determined that BP downregulates telomerase expression and decreases telomerase activity in a dose-dependent manner (Figs. 1 and 3).

The *hTERT* promoter regulatory region contains a number of potential transcription factor binding sites. Among them, the *Myc/Mad* binding sites (E-boxes) have been extensively studied.[40] It has been reported that c-Myc binds to the E-boxes in the core promoter region of *hTERT* and activates *hTERT* transcription.[41] Although BP inhibited *hTERT* expression in our study, *c-Myc* mRNA and protein expression were unaffected by BP treatment (data not shown). According to Kyo's report, there are 5 Sp1 binding sites in the *hTERT* core promoter and *hTERT* expression is much higher in cancer cells than normal cells; mutation of the *Sp1* sites resulted in substantially reduced *hTERT* transactivation.[42] In the present study, EMSA analysis indicated that BP repression of *hTERT* transcriptional activity occurs via decreased *Sp1* binding activity at the promoter. Moreover, Western blot analysis revealed that Sp1 protein expression was decreased after BP treatment,

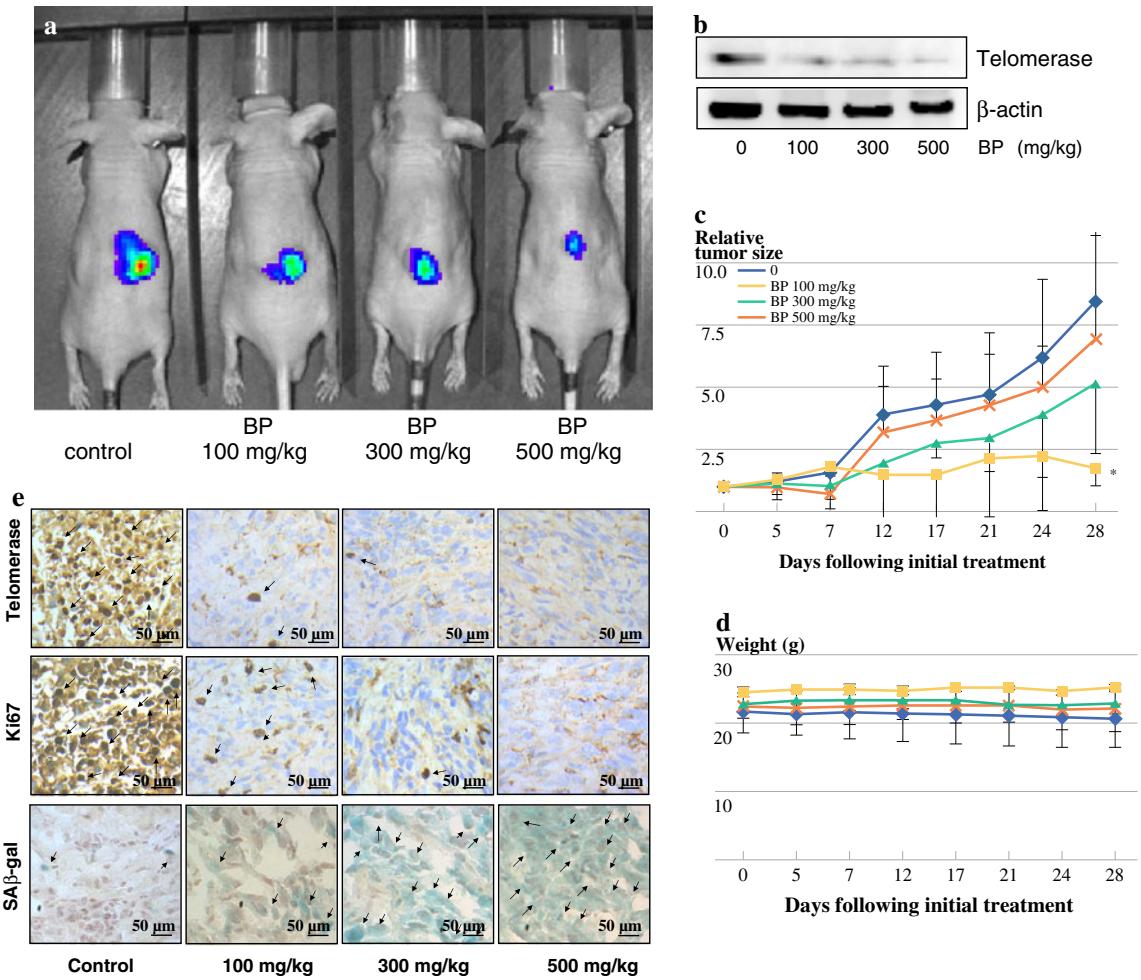


FIG. 6 BP suppresses hTERT expression in DBTRG-05MG xenograft tumors in nude mice. Human brain cancer xenografts were established by subcutaneous injection of approximately 2×10^6 GFP-DBTRG-05MG cells into the dorsal subcutaneous tissue of nude mice. After tumors reached approximately 50 mm^3, mice were given daily subcutaneous injections of vehicle alone (control group; $n = 3$) or the indicated amount of BP (treatment group; $n = 3$ each) for 5 successive days. **a** Representative images of mice from each treatment group. The volumes of the tumors were measured twice a week after tumors were detected using an IVIS imagining system. **b** Tumors were removed and homogenized for protein extraction. Samples (100 μg) were subjected to Western blot analysis using an antibody against Telomerase and β-actin was analyzed as a loading control. **c** Relative tumor volume in each treatment group ($n = 3$). Data reflect the mean ± SD. **d** Body weight ($n = 3$ per group) following treatment of BP. Data reflect the mean ± SD. **e** Paraffin-embedded tumor tissue sections (5 μm) were subjected to immunohistochemical staining (*brown*, *arrows*) using antibodies against telomerase and Ki67 (upper and middle panels; 400×). Additional tumor tissues were snap-frozen in OCT mounting medium and 12-μm sections were subjected to SA-β-gal staining (*blue*, *arrows*; *lower panels*). Representative images are shown; bar = 50 μm. *$P < .05$

correlating with reduced telomerase activity (Fig. 2a and b). Thus, the level of Sp1 expression may be a critical determinant of telomerase activity.

In this study, the viability of DBTRG-05MG cells was shown to decrease with increasing BP concentrations (Fig. 1a). As shown in our previous report, BP can promote GBM cells apoptosis through *Nur77* upregulation.[26] However, after we treated *Nur77* siRNA could only rescue 20% cell viability. In this study, we found that *hTERT* downregulation contributed partially in BP-inhibited GBM cell growth. Because cell viability is regulated by apoptotic and proliferative signals, we hypothesize that BP suppress telomerase results in cytostatic effects in GBM cells. In our result, after BP treatment, DBTRG cell's doubling time extension depends on BP concentration (Fig. 1b). In the CMV drive hTERT-HA overexpression (phTERT) GBM cells, we found the cell still undergoes apoptosis (Supplementary Fig. 1). However, after hTERT expression is restored and combined with Nur77 siRNA treatment, we found the cell viability almost rescued (Supplementary Fig. 2). This result suggests that *Nur77* and *hTERT* each take part in BP inhibiting GBM cell growth. We believe that the BP-induced cytotoxic and cytostatic effects might be contributed by *Nur77* and telomerase separately.

In order to follow the trail of ectopic *hTERT* expression, we transformed 8401 cells with the *hTERT-HA* construct (phTERT). In Counter's report, hTERT-HA has been shown to be defective for telomere lengthening, despite displaying enzymatic activity on artificial substrates.[43] In our study, chromosomes' telomeres are slightly longer in *hTERT-HA* expressing 8401 cells than the control cells (data not shown). This result agreed with Counter's report suggesting that the addition of the HA tag to hTERT may not completely disrupt the in vivo function of this protein. Interestingly, in our study exogenous hTERT-HA expression abolished BP-mediated cell-cycle arrest (Fig. 5a and b). Besides the telomere elongation, 1 possible mechanism might be that hTERT regulates a number of growth-promoting factors (i.e., Akt, STAT3) and cell-cycle regulatory components (i.e., cyclin D1) to promote cell growth.[44]

Senescence is an irreversible cell-cycle blockade. Senescent cells remain viable but do not synthesize DNA, and increased activity of senescence-associated β-galactosidase (SA-β-gal) has been reported.[45] As cells age, telomere repeats and telomere overhangs become progressively shorter until the change in telomere structure (due to critically shortened telomeres) triggers entry into senescence.[46] Culture shock induced senescence has been observed in explanted culture cells under extrinsic stresses.[47] Finally, drug-induced senescence can be induced in vitro by a number of DNA damaging drugs such as the topoisomerase I inhibitor camptothecin, the topoisomerase II inhibitor adriamycin, and the cross-linking agent cisplatin.[45,48] SA-β-gal activity has become widely recognized as a biomarker that correlates with senescence.[49] We observed that the majority of brain cancer cells treated with BP displayed intense SA-β-gal staining in association with telomerase activity (Fig. 3c). Moreover, by using colony formation assay and stained by SA-β-gal staining in 8401 phTERT transformation cells, we account the senescence colony numbers and observed a substantial decrease ($\sim$87%) in the number of senescent colonies after BP treatment, although total colony outgrowth did not increase.

Senescence depends on a number of signaling pathways that together result in a permanent and irreversible cell-cycle blockade. In these pathways, p53, p21, and p16 are the 3 major cell-cycle regulatory proteins that are associated with senescence.[50] p53 is a downstream signaling molecule that is stimulated by telomere shortening and chromosome instability, and it is used to arrest cell division and to allow repair of chromosomal damage and restore genomic stability.[51] In our previous study, we observed that BP treatment could promote the phosphorylation of p53 in the DBTRG-05MG tumor cells and increase p53 expression.[24] Moreover, the senescence associated markers, *p21* and *p16*, increased after treatment with BP. Future experiments would be necessary to determine how BP suppression hTERT induces tumor cell senescence.

Consistent with our in vitro data, a nude mice xenograft model demonstrated that BP repressed telomerase expression and inhibited tumor proliferation, resulting in tumor senescence (Fig. 6b and e). Moreover, body weight loss was not observed among mice treated with a high dose of BP (Fig. 6d) compared with the untreated group. Histological analysis of various organs (including testis and bone marrow) indicated that BP had low or no drug-related toxicity (data not shown) likely because most somatic cells, including normal brain cells, lack active telomerase. However, since both human germ cells and stem cells exhibit telomerase activity, inhibition of telomerase should reduce telomere length in these cells. Because telomerase is minimally or transiently expressed in normal tissues, including noncancerous stem cells, and because telomeres are longer in normal cells than in tumor cells, telomerase-targeted drugs will likely provide tumor specificity with minimal toxicity to normal tissues.[52] Inhibition of telomerase would be expected to cause critically short telomeres in tumor cells, leading to growth arrest or apoptosis at an earlier stage than in normal cells.[53] All of these factors indicate that cancer drugs targeted against telomerase might have an application to a wide variety of cancer types. Together, these results suggest that the *hTERT* suppressor BP might be useful for targeted cancer therapy.

In conclusion, we have demonstrated in the present study that BP downregulates telomerase activity via repression of *hTERT* mRNA expression in human glioblastoma brain cancer cells. The antiproliferative and senescence effects of BP may be due to inhibition of telomerase activity. Although the mechanism how hTERT suppression induces senescence is not clear, this study clearly suggests that BP should be further evaluated as a potential therapeutic agent for the treatment of various malignancies, including brain cancer.

ACKNOWLEDGMENT We thank Robert A. Weinberg for the pCI neo-hEST2-HA (Addgene plasmid 1782) construct. Grant: This work was supported by grants from the National Science Council of the Republic of China (96-2320-B-039-044-MY3), from the Center for Neuropsychiatry, China Medical University and Hospital, Taichung, Taiwan, and from China Medical University and Hospital, Taichung, Taiwan (DMR-99-076). This study is supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004).

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