Extended O^6-Methylguanine Methyltransferase Promoter Hypermethylation Following *n*-Butylidenephthalide Combined with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) on Inhibition of Human Hepatocellular Carcinoma Cell Growth

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Epigenetic alteration of DNA methylation plays an important role in the regulation of gene expression associated with chemosensitivity of human hepatocellular (HCC) carcinoma cells. With the aim of improving the chemotherapeutic efficacy for HCC, the effect of the naturally occurring compound *n*-butylidenephthalide (BP), which is isolated from a chloroform extract of *Angelica sinensis*, was investigated. In both HepG2 and J5 HCC cell lines, a synergistic antiproliferative effect was observed when a low dosage of BP was combined with the chemotherapeutic drug 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). BCNU is an alkylating agent, and it prompts us to examine one of DNA repair genes, O^6-methylguanine methyltransferase (MGMT). It was evident from methylation-specific polymerase chain reaction (PCR) analysis that BP/BCNU combined treatment caused a time- and concentration-dependent enhancement of MGMT promoter methylation. Overexpression of MGMT could abolish BP-induced growth inhibition in the J5 tumor cell line as measured by colony formation assay. When BP was combined with BCNU and administered, it showed significant antitumor effects in both HepG2 and J5 xenograft tumors as compared with the use of only one of these drugs. The BCNU-induced apoptosis and inhibited MGMT protein expression in HCC cells, both *in vitro* and *in vivo*, resulting from the combination treatment of BP and BCNU suggest a potential clinical use of this compound for improving the prognosis for HCCs.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide. The main curative therapies for cancer are surgery and radiation, which, in general, are only successful if the cancer is diagnosed at an early stage. Currently, conventional chemotherapy for the treatment of advanced tumors has been associated with toxicities to normal tissue and organs (*1, 2*). It is clear that new therapeutic options are necessary. Recent progress in the identification and characterization of new targets for cancer and the limited effectiveness of conventional treatment strategies have led to the development of new types of anticancer drugs. *Angelica sinensis* (Oliv.) Diels (AS), also referred to as dong quai or danggui, a traditional Chinese medicine for menopausal symptoms, has been clinically administered for several gynecological symptoms in the United States (*3, 4*). In our previous study, the chloroform extract of AS (AS-C) and n-butylidenephthalide

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Article

J. Agric. Food Chem., Vol. 58, No. 3, 2010 **1631**

(BP), derived from AS-C, both revealed dramatic antitumor effects, causing growth arrest and apoptosis of malignant brain tumors and hepatocellular carcinoma (HCC) cells *in vitro* and *in vivo* (*5−8*).

In our previous studies, we found that BP could directly injure tumor DNA and cause tumor cell arrest at the G0/G1 phase in order to repair the damage. Further, we proposed that the BP-induced growth inhibition effect might be attenuated by the MGMT (O^6-methylguanine-DNA methyltransferase) transfection assay (*6*). These observations suggest that expression of MGMT is associated with the antitumor activity of BP. It is well-known that an alkylated base adduct can be generated endogenously or through exposure to alkylating carcinogens and antitumor drugs with methylation/chloroethylating properties, such as chemotherapeutic 2-chloroethyl-*N*-nitrosourea derivatives [e.g., *N*,*N*-bis(2-chloroethyl)-*N*-nitrosurea (BCNU)]. MGMT is a recognized DNA repair protein that removes alkyl-adducts from the O^6-position of guanine in a reaction that transfers the alkyl group from the DNA to an internal cysteine residue in the MGMT, thus restoring the integrity of DNA (*9*, *10*). Increased expression of MGMT is associated with the resistance of tumor cells. On the other hand, MGMT-deficient cells showed hypersensitivity toward O^6-alkylating agents (*11*). Recently, several clinical studies have shown that glioma patients with MGMT promoter methylation responded better to treatment with radiotherapy and either BCNU or temozolomide and had improved survival compared with chemotherapy alone (*12*, *13*). Thus, finding ways of controlling MGMT expression, which could enhance the cytotoxicity of O^6-alkylating agents toward cancer cells, is of significant clinical interest.

In this study, CalcuSyn software and the combination index (CI) method were used to evaluate the interaction of n-BP and chemotherapeutic agents on HCC. It was revealed that BP has a novel role as a chemosensitizer in combination with BCNU for HCC. Further, it is interesting to observe that both HepG2 and the J5 HCC cell lines showed hypersensitivity toward BCNU, caused by the down-regulation of the responsive factors of MGMT inactivation. Combination chemotherapy using anticancer drugs achieves a better response rate, exceeding the efficacy of a single treatment. Our results distinguish the mode of action of BP as it interacts with BCNU in a higher synergistic manner and suggest a novel mechanism for understanding the downstream effectors of BP-induced growth inhibition in HCC cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human hepatocellular carcinoma cell line HepG2 (BCRC 60380) was obtained from Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). The J5 line of human hepatocellular carcinoma cells was kindly provided by Dr. M. J. Chou (Graduate Institute of Basic Medical Science, Chang Gung University). The HepG2 cells were maintained with DMEM medium containing 10% fetal bovine serum and the J5 cells were maintained with RPMI-1640 medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

Chemicals and Reagents. *n*-Butylidenephthalide (BP; molecular weight 188.23) was purchased from Lancaster Synthesis Ltd. (Newgate Morecambe, U.K.) and dissolved in DMSO to a concentration of 100 μg/mL and stored in −20 °C as a master stock solution. RPMI 1640 medium, Eagle's minimum essential medium, fetal bovine serum (FBS), penicillin, streptomycin, trypsin/EDTA, and NuPAGE Bis-Tris electrophoresis system (precast polyacrylamide mini-gel) were purchased from Invitrogen (Carlsbad, CA). RNA isolation kit was purchased from Qiagen Company (Valencia, CA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Removal reagent was from Dainippon Pharmaceutical Co. (Osaka, Japan). Annexin V-FLOUS staining kit was from Roche Molecular Biochemicals (Mannheim, Germany). Polyvinyldenefluoride (PVDF) membranes, BSA protein assay kit, and Western blot chemiluminescence reagent were purchased from Amersham Biosciences (Arlington Heights, IL).

Growth Inhibition Assay. The viability of the cells after treatment with various chemicals was evaluated using the MTT assay preformed in triplicate. Briefly, the cancer cells (5×10^3) were incubated in 96-well plates containing 200 μL of the culture medium. Cells were permitted to adhere for 12−18 h then washed with phosphate-buffered saline (PBS). Solutions were always prepared fresh by dissolving 0.2% DMSO or drugs in culture medium and then were added to HepG2 and J5 cells. After 48 h of exposure, the drug-containing medium was removed, washed with PBS, and replaced by fresh medium. The cells in each well were then incubated in culture medium with 500 μg/mL MTT for 4 h. After the medium was removed, 200 μL of DMSO and 25 μL of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well. Absorbance at 570 nm of the maximum was detected by a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The absorbance for DMSO-treated cells was considered as 100%. The results were determined by three independent experiments.

Evaluation of the Effect of BP Used in Combination with BCNU on Inhibiting Proliferation of J5 and HepG2 cells. A commercial CalcuSyn software package (Calcusyn, Biosoft, Cambridge, U.K.), was used for median-effect analysis as described by Chou and Talalay (*14*, *15*). The dose−effect curve was plotted using a logarithmic conversion of this equation to $\log(f_a/f_u) = m \log(D) - m \log(Dm)$ for each agent and then in fixed ratio combinations. In brief, J5 and HepG2 cells were seeded at a density of 5×10^3 cells/well in flat-bottomed plates (100 μL/well). After 24 h, culture medium was removed and the cells were washed with fresh FBS-free culture medium. By this method, three concentrations (6.25, 12.5, and 25 μg/mL) of BP combined with five concentrations (6.25, 12.5, 25, 50, and 100 μM) of BCNU were added into J5 and HepG2 cells. After 48 h of exposure, the drug-containing medium was removed, the cells were washed with PBS, and the medium was replaced with fresh medium. The cells in each well were then incubated in culture medium with 500 μg/mL MTT for 4 h. After the medium was removed, 200 μL of DMSO and 25 μL of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well. Absorbance at 570 nm of the maximum was detected by a PowerWave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The absorbance for DMSO-treated cells was considered as 100%. According to the survival rate, we calculated the combination index (CI) based on the formula: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$ where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) of D_1 (drug #1, for example, the BP) and D_2 (drug #2, for example, the BCNU) alone that gives x% inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the doses of D_1 and D_2 in combination that also inhibits x%. The $(D_x)_1$ and $(D_x)_2$ can be readily calculated from the Median-effect equation of Chou et al. (*14*, *15*).

RT-PCR Analysis. Cells were treated with 50 μg/mL BP for the indicated time periods (0.5, 1, 3, and 6 h). As treatment control, vehicle (DMSO) was used. After treatment, cells were washed with phosphate-buffered saline (pH 7.4), trypsinized, and scraped from the plate. Total RNA from each sample was isolated using the RNeasy Midi Kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. The concentration was calculated spectophotometrically, and RNA was adjusted to 1 μg/μL. Quality control of RNA was checked by electrophoresis and ethidium bromide staining on a 1.5% agarose gel. One microgram of total RNA from each sample was used to generate cDNA using the Omniscript RT kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. One microgram of cDNA was amplified with Taq DNA polymerase (Takara Shuzo Company, Shiga, Japan) in the presence of 1 μmol of primers (**Table 1**): DAPK1 (F), 5′-TCTACCAGCCACGGGACTTC-3′; DAPK1 (R), 5′-GCTGGCCTG-TGAGTAGACGT-3′; p53 (F), 5′-AGCGATGGTCTGGCCCCTCCT-3′; p53 (R), 5′-CTCAGGCGGCTCATAGGGCAC-3′; RASSF1 (F), 5′-TTCACCTGCCACTACCGCCTG-3′; RASSF1 (R), 5′-AGGGTGG-CTTCTTGCTGGAG-3′; MGMT (F), 5′-CCAGCAAGAGTCGTT-CACCAG-3′; MGMT (R), 5′-TCATTGCTCCTCCCACTGCTC-3′;

1632 *J. Agric. Food Chem.,* Vol. 58, No. 3, 2010

Yu et al.

Table 1. Gene-Specific Primer Sequences for Reverse-Transcription PCR and Their Expected Product Size

gene	direction	sequence	size (bp) of PCR products
DAPK1	sense	TCTACCAGCCACGGGACTTC	134
	antisense	GCTGGCCTGTGAGTAGACGT	
p53	sense	AGCGATGGTCTGGCCCCTCCT	120
	antisense	CTCAGGCGGCTCATAGGGCAC	
RASSF1	sense	TTCACCTGCCACTACCGCTG	292
	antisense	AGGGTGGCTTCTTGCTGGAG	
pl6	sense	GAATAGTTACGGTCGGAGGCC	304
	antisense	ATGGTTACTGCCTCTGGTGCC	
MGMT	sense	CCAGCAAGAGTCGTTCACCAG	134
	antisense	TCATTGCTCCTCCCACTGCTC	
E-cadherin	sense	TGATGCCCCCAATACCCCAG	209
	antisense	CTGTGGAGGTGGTGAGAGAG	
GAPDH	sense	ACCTGACCTGCCGTCTAGAA	247
	antisense	TCCACCACCCTGTTGCTGTA	

p16 (F), GAATAGTTACGGTCGGAGGCC-3′; p16 (R), 5′-ATGGT-TACTGCCTCTGGTGCC-3′; E-cadherin (F), 5′-TGATGCCCCCAA-TACCCCAG-3′; E-cadherin (R), CTGTGGAGGTGGTGAGAGAGA-G-3′; GAPDH (F), 5′-ACCTGACCTGCCGTCTAGAA-3′; GAPDH (R), 5′-TCCACCACCCTGTTGCTGTA-3′. The thermal cycling profile was composed of an initial denaturation step at 95 °C for 10 min, 35 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and 1 min of extension at 72 °C, and a final 10-min extension step at 72 °C. The intensity of bands was analyzed by GS-800 calibrated imaging densitometer (Quantity One 4.0.3 software; Bio-Rad), and levels of GAPDH were used as control.

Western Blot Analysis. Approximately 5×10^6 cells were cultured in 100-mm^2 dishes and then incubated in various concentrations of isochai-hulactone for the indicated time. The cells were lysed on ice with 200 μL of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 50 μg/mL leupeptin) and centrifuged at $13\,000 \times g$ at 4 °C for 20 min. The protein concentrations in the supernatants were quantified using a BSA protein assay kit. Electrophoresis was performed on a NuPAGE Bis-Tris electrophoresis system using 50 μg of reduced protein extract per lane. Resolved proteins were then transferred to polyvinyldenefluoride (PVDF) membranes. Filters were blocked with 5% nonfat milk overnight and then incubated with 1:500 dilutions of primary antibodies for 1 h at room temperature. Membranes were washed three times with 0.1% Tween 20 and incubated with a 1:5000 dilution of HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western Lightning chemiluminescence reagent plus and quantified using a densitometer.

DNA Extraction. Genomic DNA was extracted from ∼50 mg of colorectal tumor tissue using the Puregene genomic DNA purification kit (Gentra Systems, Inc., Minneapolis, MN). The extracted DNA was quantified spectrophotometrically (DU 640, Beckman Instruments, Inc., Fullerton, CA) before being subjected to further analysis.

Methylation-Specific PCR. Methylation in the promoter regions of *MGMT* was determined by methylation-specific PCR (MSP). In brief, genomic DNA was modified by bisulfite-mediated conversion of un-methylated cytosines using the EpiTect bisulfite kit (Qiagen, Hilden, Germany). The modified genomic DNA was amplified using primer sets designed specifically for the promoters of *MGMT* (16). All samples were amplified in a 30 μL reaction volume containing 50 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 200 mM of each primer, and 0.2 U of AmpliTaq Gold DNA poly-merase (Applied Biosystems; Roche Molecular Systems, Inc., Branchburg, NJ). The thermal cycle profile was set as follows: initial denaturation at 95 °C for 10 min, 40 cycles of 30 s denaturation at 95 °C, 45 s annealing at 56 °C, and 45 s extension at 72 °C, and a final 10 min extension at 72 °C added at the end of the cycle. PCR products were separated on 3% NuSieve agarose (3:1) gels (Cambrex Bio Science Rockland, Inc., Rockland, ME), stained with ethidium bromide, and visualized by UV transillumination.

Preparation of MGMT Vector. The pCDNA3.1 consists of a neo gene and cytomegalovirus promoter driving high-level constitutive gene expression in many mammalian cells. The coding region for human MGMT (624 bp) was inserted into the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) at *Bam*HI−*Eco*RI. Expressed MGMT protein had a molecular mass of 21 kDa. After the pcDNA−MGMT was transformed into J5 cells, the plasmid DNA was extracted using a QIAGEN Plasmid Midi kit (Qiagen, Santa Clarita, CA). The MGMT DNA was quantified with a Gene Quant spectrophotometer.

***In Vitro* Transfection.** Conditions for liposome-mediated transfec-tion were optimized for gene transfection as previously described (*8*). J5 was transfected with 2 μg of CDNA3.1−MGMT or pCDNA3.1−neo plasmids using GeneJammer reagent. After 48 h, the cells were subjected to selection for stable integrants by exposure to 800 μg/mL G418 (Invitrogen) in complete medium containing 10% FBS for 3 weeks to select the transfected cells. The cells were then assessed for overexpression of MGMT by RT-PCR analysis.

Detection of Apoptosis. Apoptosis was analyzed according to the method described by van Engeland et al. (*17*) to detect the integrity of cellular membrane and the externalization of phosphatidylserine. In brief, approximately 10^6 cells were grown in 35 mm diameter plates. The cells were treated with various herbal extracts and chemicals according to experimental design and then labeled with 10 μg/mL annexin V-FLOUS and 20 μg/mL propidium iodide (PI) prior to harvesting. After labeling, the cells were washed with binding buffer and harvested by scraping. Cells were resuspended in binding buffer at a concentration of 2×10^5 cells/mL before analysis by flow cytometry (FACScan). The data was analyzed on WinMDI V2.8 software. The percentage of cells undergoing apoptosis was determined by three independent experiments.

MGMT Activity Assay. MGMT activity was assayed by detecting removal of O^6-methylguanine using an MGMT assay kit (Sigma). In brief, cell lysates from control cells and cells transfected with MGMT (50 g protein samples) were reacted separately with 0.1 pmol of an [α-^{32}P]dTTP end-labeled 18-bp O^6-methylguanine-containing DNA substrate at 37 °C for 2 h in 150 μL of analysis buffer (50 mM Tris·HCl, pH 8.0, 0.1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol). The 18 bp oligonucleotide substrate, containing a single methyl lesion at the O^6 position of guanine within a *Pvu*II site was custom synthesized using an automated DNA synthesizer and purified (*10*). The DNA substrate was precipitated by centrifugation and air-dried. The samples were reacted with *Pvu*II for 1 h at 37 °C, and the reaction was terminated by addition of 90% formamide loading buffer. The samples were electrophoresed on a 20% denaturing gel. The gel was dried, and DNA oligonucleotides with or without cleavage were visualized by X-ray film. Densitometry analysis was done using Kodak analysis software.

Antitumor Activity *in Vivo*. Xenograft mice were used as a model system to study cytotoxicity of BP *in vivo*: the implantation of cancer cells was carried out similarly to previous reports. Female congenital athymic BALB/c nude (*nu/nu*) mice were purchased from National Sciences Council (Taipei, Taiwan), and all procedures were performed in compli-ance with the standard operating procedures of the Laboratory Animal Center of Ilan University (Ilan, Taiwan). All experiments were carried out using 6−8-week old mice weighing 18−22 g. The animals were subcuta-neously implanted with 2×10^6 HepG2 or 5×10^6 J5 cells into their backs. When the tumor reached 80−120 mm^3 in volume, animals were divided randomly into control and test groups consisting of six mice per group (day 0). Daily subcutaneous (sc) administration of BP, dissolved in a vehicle of 20% Tween 80 in normal saline (v/v), was performed from days 0 to 4 far from the inoculated tumor sites (>1.5 cm). The control group was treated with vehicle only. The mice were weighed three times a week up to days 21−28 to monitor the effects, and at the same time, the tumor volume was determined by measurement of the length (*L*) and width (*W*) of the tumor. The tumor volume at day *n* (TV$_n$) was calculated as TV (mm^3) = $(LW^2)/2$. The relative tumor volume at day *n* (RTV$_n$) versus day 0 was expressed according to the following formula: RTV$_n$ = TV$_n$/TV$_0$. Tumor regression (T/C (%)) in treated versus control mice was calculated using: T/C (%) = (mean RTV of treated group)/(mean RTV of control group) × 100. Xenograft tumors as well as other vital organs of treated and control mice were harvested and fixed in 4% formalin, embedded in paraffin, and cut in 4 mm sections for histologic study.
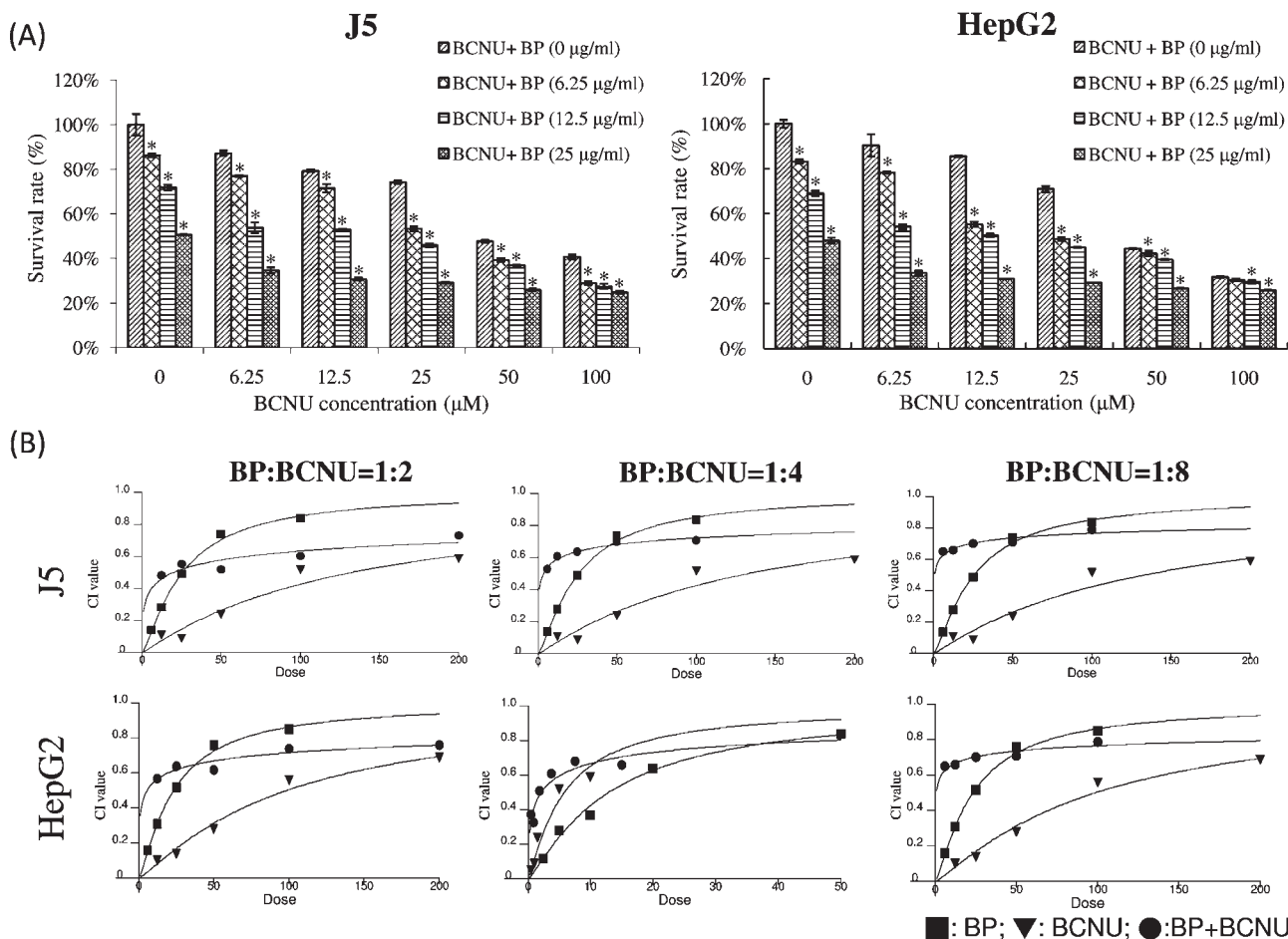
Figure 1. BP synergistically interacts with chemotherapeutic drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in inhibiting the proliferation of human hepatocellular (HCC) carcinoma HepG2 and J5 cells *in vitro*: (**A**) Both HCC cell types were treated with various concentrations of BP combined with BCNU. After 48 h of incubation, growth inhibition (IC_{50}) was determined by MTT assay. The data represent the mean and SD from three independent experiments. The ∗ indicate $P < 0.05$. (**B**) CI values for the combination treatments of BP with BCNU tested on HepG2 and J5 cells. CI values were calculated from the dose−response curve shown in panel **B** and are given in **Table 2**.

Immunohistochemical Staining. All tumor tissues (sc HepG2 and J5 tumors with or without BP treatment) were fixed in 4% formalin at 4 °C for 16 h and then embedded in paraffin. Paraffin sections were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. The sections were incubated with blocking solutions (5% milk power, 1% bovine serum albumin in phosphate-buffered saline) for 60 min at room temperature, followed by an overnight incubation with a 100-fold dilution of MGMT or caspase-3 rabbit polyclonal antibody (Cell Signaling Technology) in blocking solution. Subsequently, the immune complexes were visualized using the horseradish peroxidase-conjugated antigoat IgG secondary antibodies (1/1000 dilution; Santa Cruz Biotechnology Inc.) and LSAB2 system (DAKO, Corp., Carpinteria, CA), respectively, and then incubated for 10 min with 0.5 mg/mL diaminobenzidine and 0.03% (v/v) H_2O_2 in PBS. Finally, sections were counterstained with hematoxylin, mounted, observed under a light microscope at magnifications of 400×, and photographed.

Statistical Analysis. The data was shown as mean with standard deviation. The statistical difference was analyzed using the Student's *t* test for normal distributed values and by nonparametric Mann−Whitney U test for values of non-normal distribution. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

BP Increases the Cytotoxicity of BCNU in Hepatocellular Carcinoma J5 and HepG2 Cells, and BP Synergistically Interacts with BCNU in Both HCC Cells. BP can decrease proliferation of both HepG2 and J5 HCC cells. However, since it not known whether BP will increase sensitivity of both HepG2 and J5 HCC

Table 2. CI Values at ED_{50} of J5 and HepG2 Cells with BP/BCNU Combined Treatment

cell line	BP/BCNU		
	1:2	1:4	1:8
J5	0.29693	0.22419	0.06381
HepG2	0.34558	0.22223	0.07967

cells to other anticancer chemotherapeutic drugs, dose−response curves of BCNU and its combination with BP at different doses (6.25−25 μg/mL) were generated (**Figure 1A**). To further clarify whether enhanced anticancer chemotherapeutic drug sensitivity occurred in human cancer cells, both the HepG2 and J5 HCC cells were exposed to BP, and a CalcuSyn analysis was used to evaluate whether BP has any synergistic effect with other anticancer drugs. As shown in **Figure 1B**, HepG2 and J5 cells were simultaneously exposed to BP and alkylating agent BCNU at equipotent molar ratios for three generations to obtain CI plots. The CI values are summarized in **Table 2**. In this study, a pure compound, *n*-butylidenephthalide (BP), derived from the Chinese herb *Angelica sinensis* (Oliv.) Diels (AS), showed a synergistic effect when given simultaneously with the alkylating agent BCNU to human hepatocellular carcinoma cancer cells (**Figure 1A,B**). In contrast, no significant effect was observed in response to the combinations of BP with 5-FU or cisplatin (data not shown). These three anticancer drugs have different mechanisms

to achieve their antitumor effect. These findings suggest that BP could have specific chemotherapeutic efficacy with alkylating anticancer drugs such as BCNU in HCC cells. Although BP as a single agent has demonstrated obvious activity in solid tumors (5−8), the present findings open a new window to further consider the use of BP in solid tumors, including liver cancer. This study indicates that a low dose of BP has a synergistic antiproliferative effect in combination with the other anticancer drugs.

Down-Regulation of MGMT mRNA and Protein Expression and Enhancement of Its Promoter Hypermethylation by BP and BCNU. BP treatment inhibited the expression of *p53* and *MGMT* genes in both HepG2 and J5 cells. In addition, the expression of *DAPK1*,

Figure 2. Effects of BP on gene expression by RT-PCR. mRNA expressions of MGMT in both HCC cell types treated with 50 μg/mL of BP for 48 h as indicated. Cells were collected, and total mRNA was isolated for RT-PCR analysis. The expression of GAPDH was used as an internal control. The mRNA expression of *DAPK1, p53, RASSF1, MGMT, p16,* and *E-cadherin* genes were estimated in both cell lines.

RASSF1, E-cadherin, and *p16* genes showed no alteration in response to BP in HepG2 cells or in J5 cells (**Figure 2**). To test whether BP could induce MGMT silencing, HepG2 and J5 HCC cells were treated with 50 μg/mL BP or BCNU alone. Down-regulated *MGMT* mRNA and protein expression were determined by RT-PCR and Western blot analysis (**Figure 3B**). HepG2 and J5 HCC cells revealed a significant inhibition in MGMT protein expression measured at 2 days. It is well-known that DNA hypermethylation at the promoter CpG island plays a critical role in epigenetic silencing of tumor suppressor genes. To examine what extent of *MGMT* expression was due to its promoter methylation ability, methylation-specific PCR (MSP) analysis was used. After exposure of cells to BP for various lengths of time, it was shown that the *MGMT* promoter hypermethylation status (MSB) compared with its unmethylation status (UMSB) was up-regulated (**Figure 3A**) and resulted in down-regulation of *MGMT* mRNA and protein expression (**Figure 3A,B**). Genetic and epigenetic alterations have been identified that result in transcriptional dysregulation. The role of CpG methylation in the down-regulation of *MGMT* expression was investigated in several earlier studies, which showed a correlation between the level of *MGMT* mRNA and the extent of CpG methylation in its promoter (18−21). Our results demonstrate that BP-induced aberrant hypermethylation of the cytosine of the CpG island in the promoter region accounts for the silencing of the *MGMT* gene. Also, BP caused the inhibition of *MGMT* mRNA and protein expression (**Figure 3A,B**).

The Changes of Methylation Status and Protein Expression of MGMT and Increased Apoptosis after BP/BCNU Combination Treatment. Having established that HCC cells are methylated at the MGMT CpG island after treatment with either BP or BCNU alone, we next determined the methylation state of this gene upon treatment with a combination of BP and BCNU. MSP showed that methylation bands (MSB) were increased in the combined treatment, whereas unmethylated bands (UMSB) did not appear
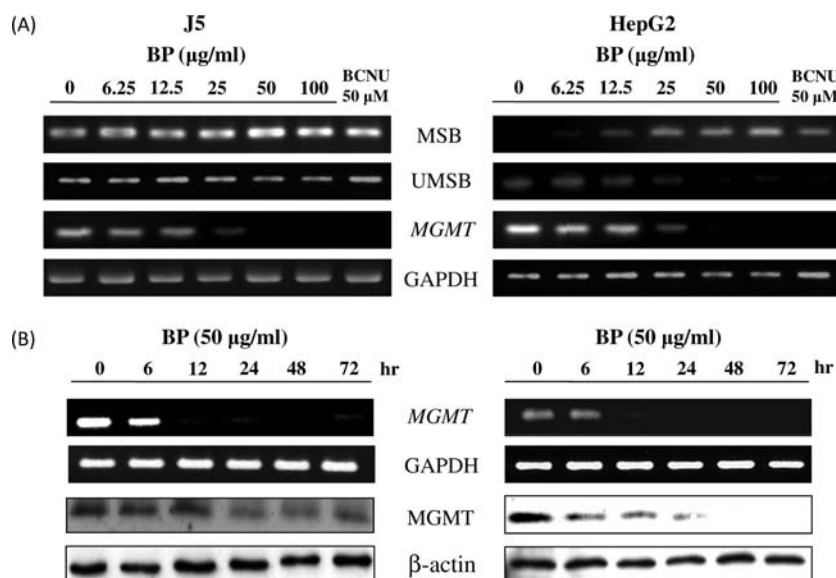

Figure 3. BP inhibited mRNA and protein expression of MGMT in HepG2 and J5 cells and induced hypermethylation of *MGMT* promoter. (**A**) Both HCC cell typess were treated with various concentrations (6.25−100 μg/mL) of BP and 50 μM BCNU for 48 h as indicated. Alternation of promoter methylation status and mRNA expression level of the *MGMT* gene was examined by bisulfite modification and methylation-specific PCR as described in Materials and Methods. The upper panel presents the methylation-specific band (MSP), the middle panel presents the unmethylation-specific band (UMSB), and the bottom panel presents the *MGMT* mRNA expression level (mRNA). (**B**) mRNA and protein expressions of MGMT in both HCC cell types treated with 50 μg/mL of BP. In each time course, cells were collected, and total mRNA and proteins were isolated for RT-PCR and Western blot analysis. The expression of *GAPDH* and β-actin were used as an internal control.
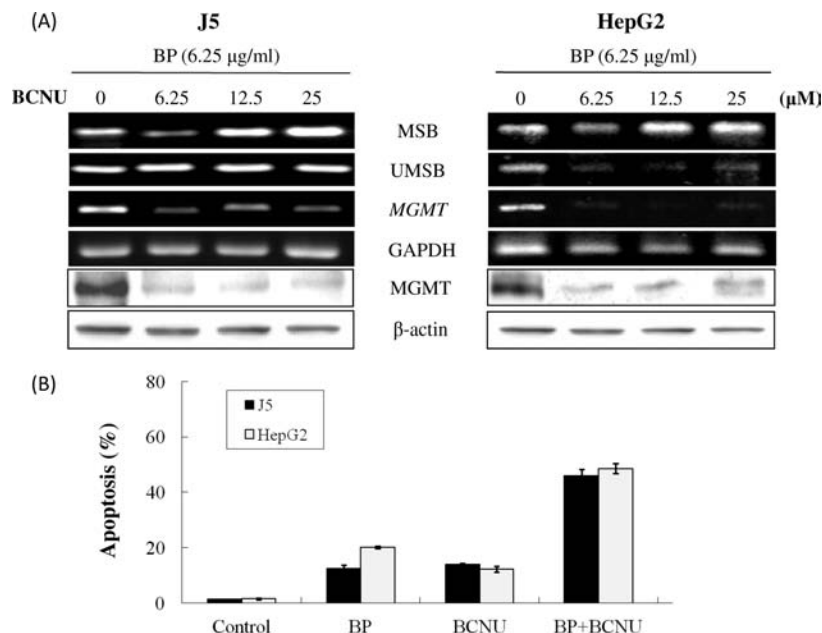
Figure 4. Additive effect of BP on *MGMT* promoter hypermethylation from BCNU in HepG2 and J5 HCC cells. (**A**) Both HCC cell types were treated with 6.25 μg/mL BP combined with various concentrations of BCNU (6.25−25 μM) for 48 h as indicated. Alteration of promoter methylation status was examined by bisulfite modification and methylation-specific PCR as described in Materials and Methods. The upper panel presents the methylation-specific band (MSP) and the bottom panel presents the unmethylation-specific band (UMSB). Bottom panels represent the *MGMT* mRNA and protein expression levels. (**B**) In HepG2 and J5 cells, 12.5 μg/mL BP induced apoptosis at a percentage of 20.5% and 17.6%, compared with the control of 2.5%. The apoptosis percentage induced by 25 μM of BCNU alone in HepG2 and J5 cells was 11.2% and 17.8%. In addition, the apoptosis percentage induced by combined exposure to BP and BCNU was 48.5% and 46.3% in HepG2 and J5 cells.

obviously changed in the two cell lines used (**Figure 4A**). Further-more, the hypermethylation of the *MGMT* promoter was con-sistent with the down-regulation of MGMT protein expression (**Figure 3B**). **Figure 4B** shows the percentage of apoptosis induced by BP or BP combined BCNU in HCC cells. In HepG2 and J5 cells, 12.5 μM BP induced apoptosis at a percentage 20.5% and 17.6%, compared with 2.5% in the control. The apoptosis rate percentage induced by 25 μM of BCNU alone in HepG2 and J5 cells was 11.2% and 17.8%. In addition, the apoptosis percentage induced by combined exposure of BP with BCNU was 48.5% and 46.3% in HepG2 and J5 cells. Therefore, we suggest that the synergistic effect demonstrated with BP and BCNU may be due to MGMT inactivation. By using MSP analysis, we found an increase in the methylation bands for the BP and BCNU combined treatment, whereas there was no obvious change in the unmethylated bands for either of the HCC cell lines (**Figure 4A**), and the hypermethylation of the *MGMT* promoter was consistent with the down-regulation of MGMT protein expression (**Figure 3B**). Furthermore, this study indicated that a dose of BP showed a synergistic antiproliferative effect in combination with BCNU.

MGMT Reversed BP-Induced Apoptosis. It has been reported that MGMT expression protects cells from the cytotoxicity caused by alkylating agents (*22−24*). In our study, J5 cells were stably transfected with an expression vector containing the full-length *MGMT* coding region in the sense orientation. A pooled population of cells obtained after selection with G418 selection reagent was then used. A Western blot of *MGMT*-transfected J5 cell populations revealed MGMT as a 21 kDa protein (**Figure 5A**). Furthermore, MGMT activity in the cells was assessed by a radiolabeled oligonucleotide clea-vage assay. As a representation of the MGMT activity assays, transfected J5 cells are shown that were positive for MGMT activity (**Figure 5B**). The MGMT-transfected J5 cells cleaved 59.6% ± 3.1% of the methyl-containing oligonucleotide,

whereas untransfected J5 cells exhibited only an 8.8% ± 0.6% cleavage (*P* < 0.01). The data from **Figure 5C** indicated that increased MGMT expression enhanced BP resistance. In the presence of 50 μg/mL BP, MGMT overexpression resulted in a significant reduction (~40%) of the clonogenic capacity of J5 cells. A higher percentage of pCDNA3.1−MGMT trans-fected cells than vector-transfected cells underwent apoptosis (**Figure 5C**).

MGMT is a unique protein that removes O^6-guanine adducts from DNA, thereby restoring the original DNA in a single step (*25*). There are no other proteins or cofactors involved in this reaction, and the MGMT protein is inactivated in the repair process. Because production of adducts at the O^6 position of guanine is the primary mechanism of cytotoxicity of some alkylating agents, silencing or inactivating MGMT results in an increase in the number of toxic or mutagenic lesions in DNA. In particular, methylating agents (i.e., temozolomide, dacarbazine, and procarbazine) and chloroethylating agents (i.e., BCNU) are known to produce toxic lesions at the O^6 position of guanine. There is compelling evidence demonstrating the importance of MGMT expression in mediating resistance to BCNU. Although the mechanism of increasing sensitivity is unclear, tumors deficient in MGMT might be treated more effectively with the combination of MGMT inhibitor O^6-benzylguanine (O^6-BG) and an alkylating agent (*26−28*). Further, it was observed that knockout of MGMT expression with small interfering RNA in human nasopharyngeal carcinoma HONE-1 cells conferred increased sensitivity to BCNU (*29*). To explore the hypothesis that MGMT may be involved in determining BP cytotoxicity, a plasmid construct containing the coding region for human MGMT was successfully trans-fected into HCC cells using liposome delivery. High-level expression of MGMT in HCC cells was confirmed by inc-reased protein expression and MGMT DNA repair activity (**Figure 5A,B**). Protection of HCC cells from the cytotoxic
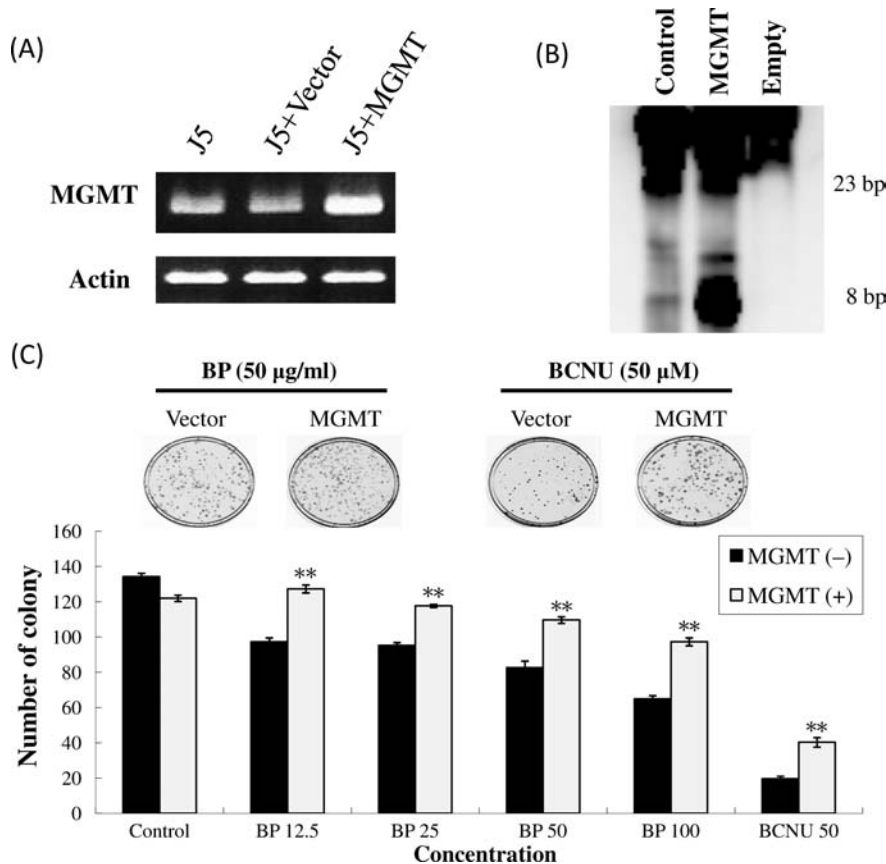
Figure 5. Effects of MGMT expression on BP-induced growth inhibition. (**A**) J5 cells were transfected with or without MGMT expression vector. Each cell was grown under G418 (500 μg/mL) for 3 weeks to select the stable clone, and the MGMT protein expression level were assayed by Western blot (control: cell extract from J5 transferred with an empty vector, MGMT: cell extract from J5 transferred with the MGME expression vector, Empty: without cell extract). (**B**) MGMT activity in transfected HCC cells. MGMT activity was measured using an 23-bp oligonucleotide assay on the sonicated cell extract (50 μg total protein/assay sample). The oligonucleotide was cleaved by MGMT to present an 8-bp fragment. MGMT represent a set of transfection experiments using pcDNA-MGMT. Densitometry of the 8-bp bands reveals that J5 cells contain some endogenous activity of MGMT (8.8 $\pm$ 0.6% cleavage), but MGMT transfected cells have significantly augmented MGMT activity (59.6 $\pm$ 3.1% cleavage), **P < 0.01. (**C**) MGMT has protective effect on BP-induced cytotoxicity of J5 cells. Both HCC cells with or without MGMT transfection were incubated in the presence of BP (0−100 μg/mL) for 24 h and the grown in culture medium for 2 weeks. The colonies were stained with 2% methyl blue solution. The results are representative of three different experiments. Cells treated with 50 μM BCNU in both HCC cells used as positive control.

effects of BP was verified by using colony formation assays and transfection viability assays. We showed that BP induced the growth arrest and apoptosis in HCC cells through the down-regulation of the protein MGMT, and our study suggests that MGMT is a critically important protein capable of reducing BP toxicity to HCC cells (**Figure 5C**).

BP/BCNU Combined Enhanced *in Vivo* Growth Inhibition of HepG2 and J5 Xenografts in Nude Mice. To evaluate the anti-tumor activity of BP, BCNU, and BP combined with BCNU *in vivo*, human liver cancer xenografts were established by sc injection of approximately 2×10^6 HepG2 and 5×10^6 J5 cells into the dorsal subcutaneous tissue and cells on the backs of nude mice. After the tumors reached approximately 100−250 mm³, the mice were randomized into vehicle control and treatment groups (six animals each) and given a daily sc injection of either 50 mg/kg BP alone or 100 mg/kg BP alone or 4 mg/kg BCNU alone or 8 mg/kg BCNU alone or 50 mg/kg BP combined with 4 mg/kg BCNU for five successive days. Significant suppression of tumor growth was observed in the BP/BCNU combined-treatment groups compared with the control group (**Figure 6A**). In addition, inhi-bition of MGMT protein in the developing tumor was confirmed by Western blot analysis in BP/BCNU combination-treated tumor cells. Down-regulation of MGMT and up-regulation of

caspase-3 expression were observed in the human HepG2 and J5 tumor tissues treated with BP/BCNU combined relative to the control group at day 10 after treatment (**Figure 6B**).

In conclusion, our data indicate that epigenetic modification may involve MGMT silencing and the depletion of MGMT in HCC cells, resulting in an increase in the growth inhibitory effect of the combination of BP and BCNU. Moreover, an antitumor effect of BP in a nude mouse xenograft animal model has been reported (7) that indicates that expression of MGMT is asso-ciated with the antitumor activity of BP. MGMT was highly inhibited *in vivo* after treatment with a combination of BP and BCNU. Together, these results suggest that the antitumor activity of BP is mediated via MGMT and that MGMT might play a role in the antitumor effect of BP in HCC cells. Furthermore, the synergistically cytotoxic effect caused by BP and BCNU has been shown to be directly associated with drug-induced *MGMT* promoter hypermethylation in HCC cells. This phenomenon should lead to greater inhibition of protein expression of MGMT and enhanced apoptosis. Our study demonstrated for the first time that BP is able to suppress the expression of the MGMT gene, thus causing a synergistic interaction with BCNU. These findings provide an explanation regarding why the combination of BP and BCNU in clinical settings results in better objective
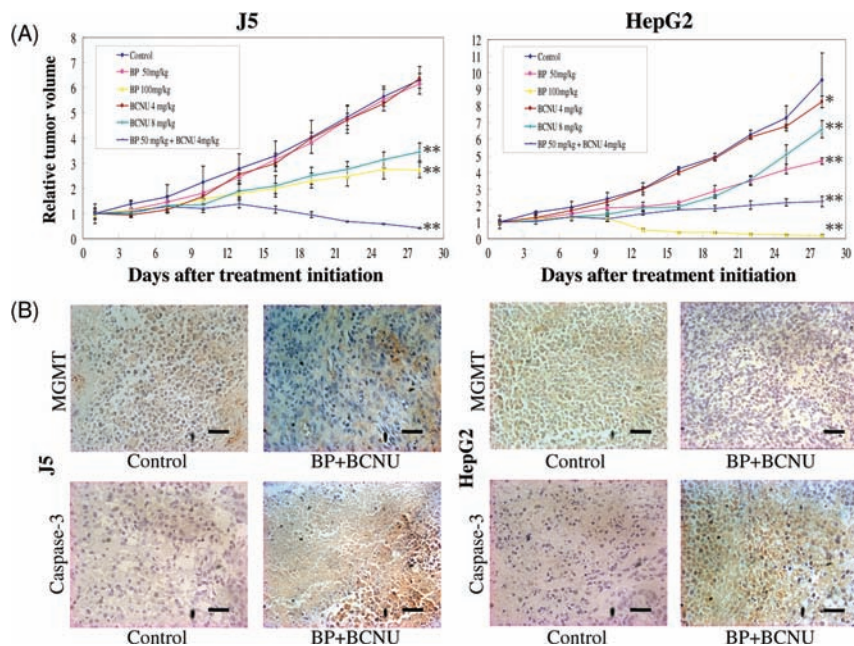
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J. Agric. Food Chem., Vol. 58, No. 3, 2010 **1637**

Figure 6. Enhancement of efficacy of BCNU in HCC xenografts in Balb/c nude mice with BP in nude mice injected with approximately 2 × 10⁶ HepG2 or 5 × 10⁶ J5 cells into the dorsal subcutaneous tissue. (**A**) When the tumor volumes reached 100−250 mm³, HepG2 and J5 tumor-bearing mice were administered sc vehicle control (◇), 50 mg/kg BP (□),100 mg/kg BP (△), 4 mg/kg BCNU (○), 8 mg/kg BCNU (+), and 50 mg/kg BP combined with 4 mg/kg BCNU (−) on days 0−4 for 5 days. The panel shows for both HCC cell types relative tumor volume of control and therapeutic groups. The data represent the mean and SD from three independent experiments; * indicates $P < 0.05$, and ** indicates $P < 0.01$. (**B**) Expressions of MGMT and caspase-3 in HepG2 and J5 xenografts. Nude mice were injected with approximately 2 × 10⁶ HepG2 or 5 × 10⁶ J5 cells into the dorsal subcutaneous tissue. When the tumor volumes reached 50−100 mm³, HepG2 and J5 tumor-bearing mice were administered sc vehicle control or 50 mg/kg BP combined with 4 mg/kg BCNU for 5 days. Five days later, tumors were homogenized for protein extraction. Representative photographs show sections of the control group and BP/BCNU combined group HepG2 and J5 tumors, immunohistochemically stained with MGMT and caspase-3 rabbit polyclonal antibodies. The MGMT and cleaved caspase-3 positive cells were stained brown (400×). Scale bars = 100 μm.

response than single-use alone. In addition, this study also supports a new indication to treat refractory hepatocellular carcinoma patients with BP and BCNU.

ABBREVIATIONS

MGMT, O⁶-methylguanine methyltransferase; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; RT-PCR, reverse transcription polymerase chain reaction; MSP, methylation-specific PCR; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide; PVDF, polyvinyldenefluoride; BP, butylidenephthalide;

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