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(Z)-Butylidenephthalide Restores Temozolomide Sensitivity to Temozolomide-Resistant Malignant Glioma Cells by Downregulating Expression of the DNA Repair Enzyme MGMT

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Abstract: Temozolomide, an alkylating agent against high-grade astrocytomas such as GBM (Glioblastoma Multiforme), is the leading compound in a new class of antitumor drugs. Altered methylation by MGMT (O6-methylguanine-DNA-methyltransferase) leads to temozolomide-resistant GBM. Thus, inhibition of MGMT activity is necessary for temozolomide effectiveness. Here we show a synergistic cytotoxic effect on the GBM cell line 8901 with a low dose of (Z)-butylidenephthalide combined with temozolomide. Using CalcuSyn software to analyze the combination index of (Z)-butylidenephthalide and temozolomide, we observed significant synergism in decreasing MGMT expression. We next investigated the effect of (Z)-butylidenephthalide on a temozolomide-resistant cell line GBM22-TMZ and examined changes in MGMT. The combination of 50 μM or 100 μM (Z)-butylidenephthalide with 1,600 μM temozolomide yielded a combination index of 0.649 or 0.581 (an index < 1 indicates synergism), indicating that increasing (Z)-butylidenephthalide enhanced the synergism. In addition, (Z)-butylidenephthalide enhanced MGMT promoter methylation and decreased MGMT expression in a dose-dependent manner. Using a GBM22-TMZ xenograft tumor model, (Z)-butylidenephthalide combined with temozolomide showed significant antitumor effects compared with a single drug. Finally, a survival study with Kaplan-Meier statistical analysis demonstrated a significant benefit of the (Z)-butylidenephthalide/temozolomide combination compared to temozolomide alone. In summary, the combination of (Z)-butylidenephthalide and temozolomide shows potential clinical relevance for treating temozolomide-resistant GBM.

Key words: Z-butylidenephthalide (Bdph), MGMT, temozolomide, GBM, temozolomide-resistant GBM.

1. Introduction

Malignant brain tumors account for about 2% of all malignant neoplasms and are difficult to prevent because no specific carcinogenic factor has been identified for this disease. High-grade gliomas, which include anaplastic gliomas (WHO grade III) and glioblastoma multiforme (WHO grade IV), are the

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most common types of primary brain tumors in adults [1, 2].

Because malignant brain tumor cells spread and grow within normal tissues, they are not easy to eradicate with surgery, and thus therapy for brain cancer must include chemotherapy [3, 4].

However, the blood-brain barrier prevents access of most anti-cancer drugs to brain tumor cells. Thus, the chemotherapeutic effect is limited [5, 6], and the average lifespan of patients with terminal brain cancer is usually no more than one year [7].

Temozolomide is one of the commonly used drug to treat malignant brain tumors [8]. Temozolomide is an imidazole tetrazine-type oral chemotherapeutic that effectively inhibits tumor growth [9]. Temozolomide is particularly effective against gliomas (including GBM and anaplastic astrocytoma) [10, 11]. The toxicity of temozolomide is high, and it produces side effects such as nausea, vomiting, headache, fatigue, anorexia, etc. [12]. Furthermore, tumor cells easily develop resistance to temozolomide, and therefore its clinical usefulness is quite limited [13].

Temozolomide is a methylating agent. By methylating the oxygen at position 6 of guanines in DNA, temozolomide may distort the double-stranded DNA structure to inhibit DNA replication, leading to cancer cell death. However, this methylation is inhibited in cancer cells by MGMT, which removes abnormal methylation in cells, thus weakening the efficacy of temozolomide [13, 14]. A study by Ranson et al. revealed that the efficacy of temozolomide improves greatly when MGMT expression is inhibited in patients [15]. Therefore, the authors proposed the the efficacy of temozolomide resistant could be restored by inhibiting MGMT expression.

The compound *n*-butylidenephthalide, isolated from *Angelica sinensis*, inhibits telomerase activity and induces Nur77-mediated tumor cell apoptosis [16, 17]. This compound can potentially be used to treat various tumors such as GBM and breast cancer [18, 19]. Moreover, *n*-butylidenephthalide also inhibits MGMT expression in glioblastoma multiforme [20]. Here the authors report that, compared with (E)-butylidenephthalide (*trans*-butylidenephthalide), (Z)-butylidenephthalide (*cis*-butylidenephthalide; z-BP) has a higher capacity to inhibit MGMT expression and enhance the sensitivity of brain tumor cells to temozolomide. Simultaneously, (Z)-butylidenephthalide combined with temozolomide had a synergistic anti-tumor effect in a xenograft mouse model. According to these results, (Z)-butylidenephthalide provided excellent efficacy against brain cancer and further reduced the necessary dosage of temozolomide.

2. Materials and Methods

2.1 Chemicals and Reagents

Z-butylidenephthalide (BP; molecular weight 188.23) was purchased from Lancaster Synthesis Ltd. (NewgateMorecambe, U.K.). The RNA isolation kit was purchased from Qiagen Company (Valencia, CA). DMSO, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetr-azolium bromide) and horseradish peroxidase–conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). The primary antibody used was rabbit monoclonal antibodies against human MGMT (Cell Signaling Technology, Danvers, MA). Polyvinylidene fluoride membranes, BSA protein assay kit and western blot chemiluminescence reagent were purchased from Amersham Biosciences (Arlington Heights, IL).

2.2 Cell Lines and Cell Culture

The human GBM cell lines 8401, 8901, DBTRG and G5T/VGH were obtained from Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). The GBM22 and GBM22-TMZ cell lines were a kind gift from Plummer ER and Sarkaria JN.The cells were maintained in DMEM containing 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3 Growth Inhibition Assay

The viability of the celllines after treatment with various chemicals was evaluated using an MTT assay preformed in triplicate. Briefly, the cancer cells (5×10^3) were incubated in 96-well plates containing 200 μL of culture medium. Cells were allowed to adhere for 12-18 h and then washed with PBS (phosphate-buffered saline). After 48 h, drug-containing medium was removed, cells were washed with PBS, and fresh medium was added. The cells in each well were then incubated in culture medium containing 500 μg/mL MTT for 4 h. Absorbance at 570 nm was detected with a PowerWave X Microplate ELISA Reader (Bio-TeKInstruments, Winoski, VT).

2.4 Evaluation of the Effect of z-BP in Combination with Temozolomide on Inhibiting Proliferating Cells

A commercial software package (Calcusyn, Biosoft, Cambridge, U.K.) was used for median-effect analysis as described by Chou and Talalay [21, 22]. The dose-effect curve was plotted using a logarithmic conversion of this equation to log (fa/fu) = m log (D) − m log (Dm), for each agent , resulting in fixed ratio combinations. Briefly, cells (5×10^3 per well in 100 μL) were seeded in flat-bottomed 96-well plates. After 24 h, culture medium was removed, and the cells were washed with fresh fetal bovine serum–free culture medium. Using this method, four concentrations (25 μM, 50 μM, 100 μM and 200 μM) of z-BP combined with six concentrations (100 μM, 200 μM, 400 μM, 800 μM, 1,000 μM and 1,200 μM) of temozolomide were added to 8901 cells, and five concentrations (50 μM, 100 μM, 200 μM, 400 μM and 600 μM) of z-BP combined with six concentrations (100 μM, 200 μM, 400 μM, 800 μM, 1,600μM and 3,200 μM) of temozolomide were added to GBM22-TMZ cells. After 48 h, 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 containing 500 μg/mL MTT for 4 h. After the medium was removed, 200 μL DMSO and 25 μL glycine buffer were added to each well. Absorbance at 570 nm was detected with a PowerWave X Microplate ELISA Reader. According to the survival rate, we calculated the combination index (CI) based on the formula: $CI = (D)1/(Dx)1 \text{ þ } (D)2/(Dx)2$ where $(Dx)1$ and $(Dx)2$ in the denominators are the doses (or concentrations) of D1 (drug #1, for example, z-BP) and D2 (drug #2, for example, temozolomide) alone that give x% inhibition, whereas (D)1 and (D)2 in the numerators are the doses of D1 and D2 in combination that also inhibit by x%. (Dx)1 and (Dx)2 can be readily calculated from the median-effect equation as described by Chou et al. [21, 22].

2.5 RT-PCR Analysis

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 spectrophotometrically, and the RNA concentration was adjusted to 1 μg/μL. RNA quality control was qualitatively 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 primers:

MGMT (F), 5′-CCAGCAAGAGTCGTTCACCAG-3′;
MGMT (R), 5′-TCATTGCTCCTCCCACTGCTC-3′;
GAPDH (F), 5′-ACCTGACCTGCCGTCTAGAA-3′;
GAPDH (R), 5′-TCCACCACCCTGTTGCTGTA-3′.

The thermal cycling conditions were 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.

2.6 DNA Extraction and MSP (Methylation-Specific PCR)

Genomic DNA was extracted using the QIAmp DNA Mini kitqAZ (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA modification was done with the sodium bisulfide reaction using an EpiTect® Bisulfite kit (Qiagen, Hilden, Germany). Modified genomic DNA was amplified using primer sets designed specifically for the MGMT promoter [23]. Primers for the methylated and unmethylated alleles were 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (sense; methylated), 5′-GCACTCTTCCGAAAACGAAACG-3′ (anti-sense; methylated), 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (sense;unmethylated), and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (anti-sense; unmethylated). The thermal cycle conditions were: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, 40 cycles of annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and a final 10-min extension at 72 °C at the end of the cycle.

2.7 Western Blot Analysis

Approximately 5×10^6 cells were cultured in 100 mm^2 dishes and then incubated in various concentrations of z-BP and temozolomide for various periods. The cells were lysed on ice with 200 μL lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 50 μg/mL leupeptin) and centrifuged at 13,000 × g at 4 °C for 20 min. Electrophoresis was performed on a NuPAGEBis-Tris electrophoresis system using 50 μg of reduced protein extract per lane. Resolved proteins were then transferred to polyvinylidene fluoride membranes, which were blocked with 5% nonfat milk in PBST overnight at 4 °C and then incubated with 1:500 dilutions of primary antibodies for 1 h at room temperature. All proteins were detected using the Western Lightning Chemiluminescence Reagent Plus (Amersham Biosciences, Arlington Heights, IL) and quantified using a densitometer.

2.8 Antitumor Activity in Vivo

Female congenital athymic BALB/c nude (nu/nu) mice were purchased from the National Sciences Council (Taipei, Taiwan), and all procedures were performed in compliance with the standard operating procedures of the Laboratory Animal Center of China Medical University (Taichung, Taiwan). The animals were subcutaneously implanted with 2×10^6 GBM22-TMZ cells into their backs. When the tumor reached a volume of 80-120 mm^3, animals were divided randomly into control and test groups consisting of six mice per group (day 0). Temozolomide was purchased from the Mayo Clinic Pharmacy, suspended in Ora-plus (Paddock Laboratories), and administered by oral gavage at 66 mg/kg/d for 5 d. z-BP was administered via subcutaneous injection at 50 mg/kg/d or 100 mg/kg/d for 5 d, coinciding with temozolomide therapy and given 1 h before temozolomide dosing. The control group was treated with vehicle only. For analysis tumor regression, the mice were weighed three times a week up to days 30, and at the same time, the relative tumor volume at day n (RTVn) versus day 0 was calculated as: $RTVn = TVn/TV_0$. (T/C (%)) in treated versus control mice was calculated as: T/C (%) = (mean RTV of treated group)/(mean RTV of control group) × 100. In addition, for analysis survival, nude mice were sacrificed when tumor volume exceeded 1,000 mm^3 and that day was used as the final survival day.

2.9 Statistical Analysis

The data are shown as the mean ± standard deviation. Statistical differences were analyzed using the Student's *t*-test for normally distributed values and with a nonparametric Mann-Whitney U-test for values

with non-normal distributions. Values of $p < 0.05$ were considered significant. Survival analysis was according to Kaplan-Meier statistical analysis.

3. Results

3.1 z-BP Increases the Cytotoxicity of Temozolomide in Malignant Glioma 8901 Cell Line and Synergistically Interacts with Temozolomide in both 8901 Cell Line and GBM22-TMZ Cell Line

First, we determined the viability of the brain tumor cell lines after z-BP and temozolomide treatment. The IC_{50} of temozolomide on GBM22-TMZ cell line (temozolomide-resistant cell line) was over 3,200 μM, and the IC_{50} of z-BP on GBM22-TMZ cell line was 450.9 μM (Table 1).

To further clarify whether enhanced anticancer chemotherapeutic drug sensitivity occurred in human brain cancer cells, both 8901 (temozolomide-sensitive) and GBM22-TMZ cell line were exposed to z-BP and temozolomide (Table 2), and CalcuSyn analysis was used to evaluate whether z-BP had a synergistic effect with temozolomide (Table 3). In 8901 cell line, the combination of 50 μM z-BP (IC_{50} = 218.62 μM) and 200 μM temozolomide (IC_{50} = 1201.6 μM) yielded a combination index of 0.841, indicating a synergistic effect. In addition, the combination of 100 μM z-BP and 200 μM temozolomide yielded a combination index of 0.610. This result indicates that a low dose of z-BP has a synergistic cytotoxic effect in combination with temozolomide although high dose has much effect (Table 4).

In the temozolomide-resistant cell line GBM22-TMZ, the combination of 50 μM z-BP and 1,600 μM temozolomide yielded a combination index of 0.649. The combination of 100 μM z-BP and 1,600 μM temozolomide yielded a combination index of

Table 1 Toxicity of temozolomide and (Z)-butylidenephthalide on various brain cancer cell lines.

Brain cancer cell line	IC_{50} value (μM) of temozolomide	IC_{50} value (μM) of (Z)-butylidenephthalide
DBTRG	1,658.6	531.9
8401	831.3	398.9
8901	1,201.6	218.62
G5T/VGH	1,660.1	127.6
GBM22-TMZ	>3,200	450.9

Table 2 (Z)-butylidenephthalide increases the cytotoxicity of temozolomide in the brain cancer cell line 8901.

Active ingredient	IC_{50} (μM)
Temozolomide	1,201.6
Temozolomide + (Z)-butylidenephthalide (25 μM)	992.3
Temozolomide + (Z)-butylidenephthalide (50 μM)	866.8
Temozolomide + (Z)-butylidenephthalide (100 μM)	685.5
Temozolomide + (Z)-butylidenephthalide (200 μM)	582.5

Table 3 The synergistic effect of the combination of (Z)-butylidenephthalide and temozolomide to kill the brain cancer cell line 8901.

Combination index in GBM 8901				
z-BP(μM) / TMZ(μM)	25	50	100	200
100	1.777	1.017	**0.846**	**0.951**
200	1.708	**0.841**	**0.610**	**0.991**
400	1.361	**0.902**	**0.776**	**0.922**
1,000	**0.950**	**0.839**	**0.748**	**0.827**
1,200	**0.455**	**0.242**	**0.332**	**0.369**

Bold font: synergistic combinations. CalcuSyn software was used to draw a reaction curve for the drug dosages, and the combination index was calculated to analyze the pharmacokinetic properties of (Z)-butylidenephthalide and temozolomide and their synergism, additivity or antagonism.

Table 4 The synergistic effect of the combination of (Z)-butylidenephthalide and temozolomide to inhibit the temozolomide-resistant brain cancer cell line GBM22-TMZ.

Combination Index in GBM22-TMZ					
z-BP(μM) / TMZ(μM)	50	100	200	400	600
100	1.713	1.578	2.911	1.026	**0.622**
200	1.168		2.898	1.176	**0.601**
400	1.438	1.776	2.105	**0.872**	**0.604**
800	**0.894**	**0.881**	**0.961**	**0.776**	**0.619**
1600	**0.649**	**0.581**	**0.725**	**0.513**	**0.622**
3200	**0.198**	**0.223**	**0.359**	**0.569**	**0.632**

Bold font: synergistic combinations. Because we did not know if z-BP would increase the sensitivity of GBM22-TMZcells to temozolomide, dose-response curves for temozolomide (100 μM, 200 μM, 400 μM, 800 μM, 1,600 μM and 3,200 μM) and in combination with z-BP (50 μM, 100 μM, 200 μM, 400 μM and 600 μM) were generated, and CalcuSyn was used to evaluate whether z-BP had a synergistic effect. z-BP and the alkylating agent temozolomide were used at equipotent molar ratios for three times to obtain the CI plots.

0.581, indicating greater synergistic effect for this dosage combination (Table 4). Increasing the concentration of z-BP enhanced the synergistic effect, indicating that z-BP resensitized the temozolomide-resistant cell line to temozolomide.

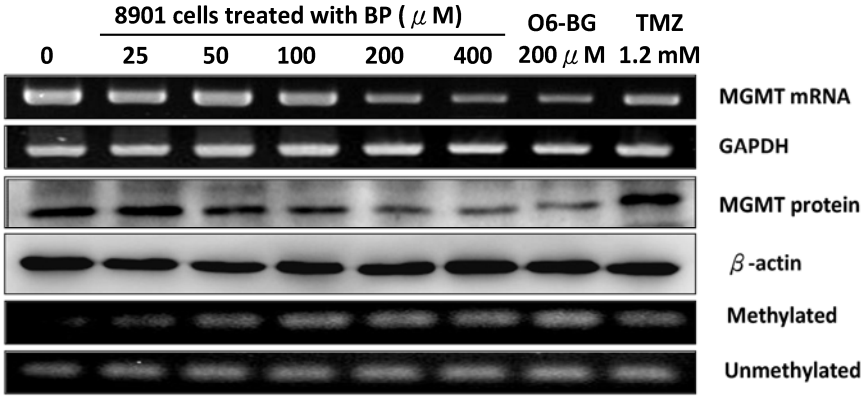
3.2 Downregulation of MGMT mRNA and Protein Expression and Enhancement of Promoter Hypermethylation by z-BP Alone or Synergistically with Temozolomide in 8901 Cell Line

To test whether z-BP induced MGMT silencing, 8901 cell line were treated with various concentrations of z-BP alone. To examine to what extent MGMT expression was due to its promoter methylation, MSP analysis was used. After exposure of cells to various concentrations of z-BP, the MGMT promoter hypermethylated status (MSB) was upregulated compared with its unmethylated status (UMSB), resulting in significantly inhibition of MGMT mRNA and protein expression in a dose-dependent manner (Fig. 1). Thus, z-BP-induced aberrant hypermethylation of the cytosines in the CpG island in the promoter region accounted for the silencing of the *MGMT* gene.

Next, we examined the methylation status of *MGMT* following treatment of 8901 cell line with a combination of z-BP and temozolomide. The cells were treated with z-BP (25 μM, 50 μM, 100 μM, 200 μM and 400 μM) and temozolomide (400 μM). MSP showed that methylated bands were increased with the combined treatment, whereas unmethylated bands decreased in a dose-dependent manner (Fig. 2). The hypermethylation of the MGMT promoter was dose dependent and accompanied by downregulation of MGMT protein (Fig. 2). The methylation of MGMT by z-BP was first apparent at 25 μM, similar to what is seen in Fig. 1.

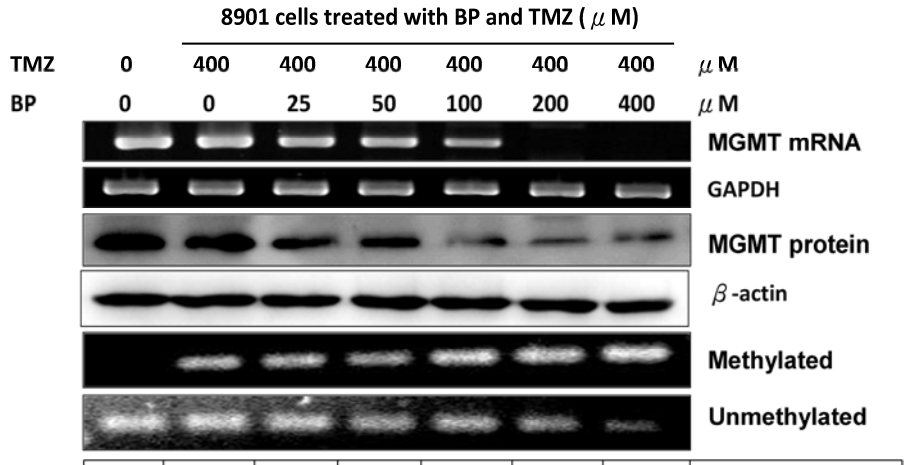
3.3 Changes in the Methylation Status of the MGMT Promoter and Protein Expression After z-BP Alone or in Combination with Temozolomide in Temozolomide-Resistant GBM22-TMZ Cell Line

After we had established that 8901 cell line (non-temozolomide resistant) were methylated at the MGMT CpG island after treatment with either z-BP alone or in combination with temozolomide, we next



1.0	0.86	0.90	0.67	0.35	0.48	0.70	0.72	MGMT mRNA
1.0	1.2	0.9	0.8	0.5	0.5	0.4	1.1	MGMT protein
1.0	5.11	9.96	15.18	13.89	11.61	16.37	10.98	Methylated
1.0	1.47	1.45	1.33	1.40	1.41	1.42	1.29	Unmethylated

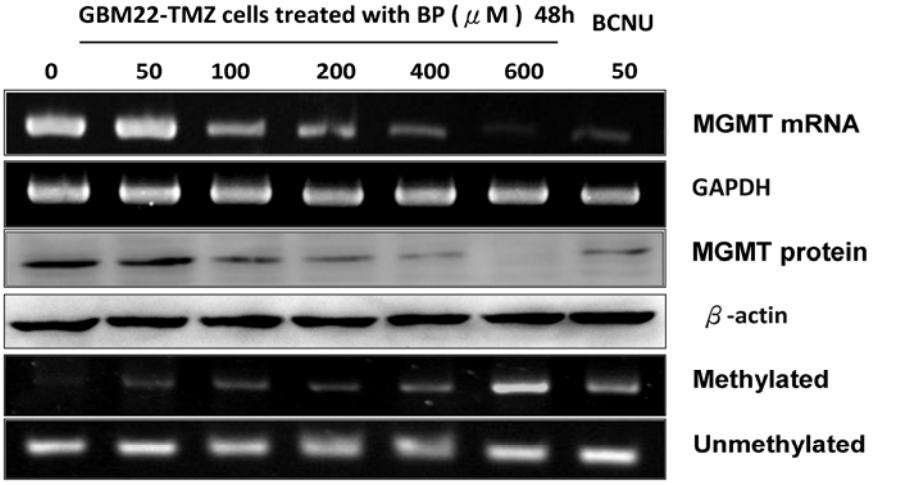
Fig. 1 Analysis of the methylation status of the *MGMT* promoter, messenger RNA and protein expression of MGMT in 8901 cell line treated with z-BP. To test whether z-BP induced MGMT silencing, 8901 GBM tumor cells were treated with z-BP (25 μM, 50 μM, 100 μM, 200 μM and 400 μM), O6-BG (positive control), or with 1.2 mMtemozolomide alone. Downregulation of MGMT mRNA and protein expression were determined by RT-PCR and western blot analysis, respectively, starting with 25 μM z-BP.



1	0.99	0.87	0.85	0.72	0.11	0.0	MGMT mRNA
1.0	0.9	0.7	0.6	0.5	0.3	0.5	MGMT protein
0.0	1.0	1.03	0.88	1.46	1.47	1.69	Methylated
1	1.2	1.11	0.96	1.01	0.76	0.25	Unmethylated

Fig. 2 Analysis of the methylation status of the *MGMT* promoter, messenger RNA and protein expression of MGMT in 8901 cell line treated with z-BP and temozolomide. We determined the methylation status of MGMT following treatment of the 8901 cell line with a combination of z-BP and temozolomide. 8901 GBM tumor cell lines were treated with z-BP (25 μM, 50 μM, 100 μM, 200 μM and 400 μM) and with temozolomide (400 μM).

determined the methylation state of this gene following treatment with z-BP alone in temozolomide-resistant GBM22-TMZ cell line. MGMT protein expression was significantly downregulated in a dose-dependent manner owing to increased CpG methylation in the promoter (Fig. 3).



1.0	1.1	0.6	0.5	0.3	0.1	0.2	MGMT mRNA
1.0	0.8	0.4	0.3	0.1	0.0	0.6	MGMT protein
1.0	4.8	7.4	6.3	8.6	22.0	18.6	Methylated
1.0	1.0	1.0	1.1	1.0	1.1	1.0	Unmethylated

Fig. 3 Analysis of the methylation status of *MGMT* in GBM22-TMZ cell line treated with z-BP. To test whether z-BP induced *MGMT* silencing, GBM22-TMZ tumor cells were treated with z-BP (50 μM, 100 μM, 200 μM, 400 μM and 600 μM) or BCNU (positive control). Downregulation of MGMT mRNA and protein expression were determined with RT-PCR and western blot analysis, respectively.

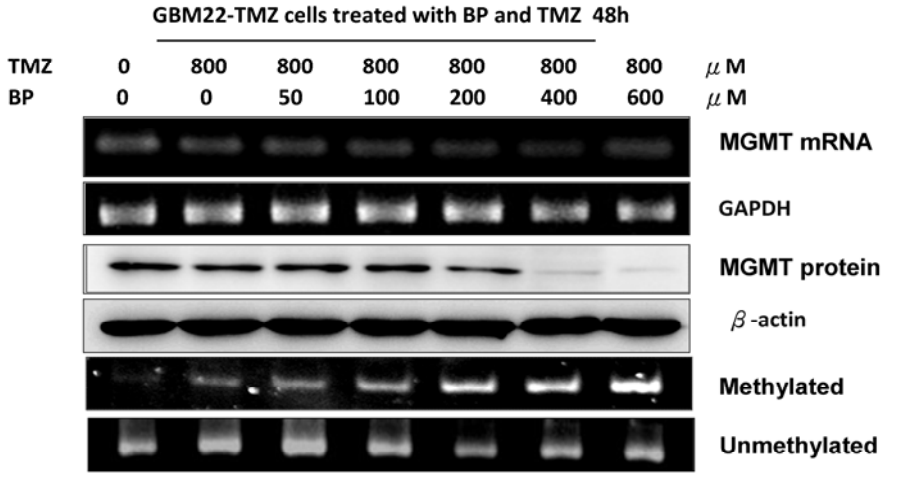
We also examined the effects of z-BP in combination with temozolomide on the methylation status of the MGMT promoter in GBM22-TMZ cell line. MSP showed that methylated bands increased with the combined treatment, whereas unmethylated bands were not obviously changed. Hypermethylation of the MGMT promoter was also consistent with the downregulation of MGMT protein (Fig. 4) in a z-BP dose-dependent manner. We also demonstrated that z-BP, but not e-BP, affected MGMT protein expression in either cell lines (Fig. 5).

3.4 Combination of z-BP and Temozolomide Enhances in Vivo Growth Inhibition and Prolongs the Survival of Nude Mice Harboring GBM22-TMZ Xenografts

To evaluate the antitumor activity of z-BP, nude mice were given xenograft transplants of GBM22-TMZ cell line and then treated with z-BP combined with temozolomide. Tumor growth was significantly suppressed in the z-BP/temozolomide combined-treatment groups compared with groups given z-BP alone, temozolomide alone or vehicle control (Fig. 6, $p < 0.05$). Survival analysis showed significantly prolonged survival in the combination groups (Fig. 7, $p < 0.05$).

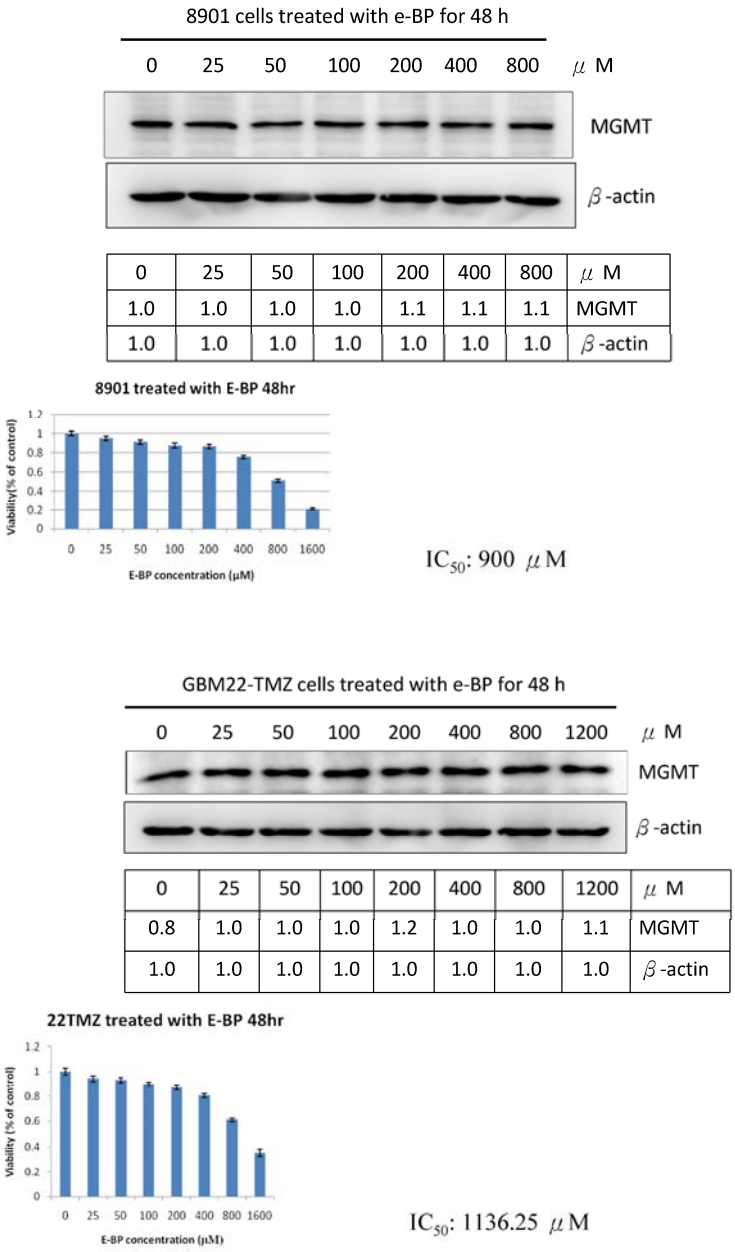
4. Discussion

Malignant gliomas (GBM and anaplastic astrocytoma) occur more frequently than other types of primary central nervous system tumors, with a combined incidence of 5-8 per 100,000 persons. Even with aggressive treatment that includes surgery, radiation and chemotherapy, the median reported survival is less than 1 year [24]. Temozolomide, a new drug, has shown promise in treating malignant gliomas and other tumors that are difficult to treat. In general, however, high MGMT protein expression in tumor samples is associated with temozolomide resistance in patients with GBM, although this association is not completely predictive of individual tumor response [25]. MGMT expression is commonly suppressed in tumors by CpG methylation in the MGMT promoter, and thus tumor MGMT hypomethylation is associated with temozolomide



1.0	0.78	0.74	0.6	0.49	0.4	0.54	MGMT mRNA
1.0	0.8	0.9	0.8	0.6	0.0	0.0	MGMT protein
1.0	2.8	4.0	5.1	7.8	7.7	9.7	Methylated
1.0	1.5	1.7	1.4	1.0	1.2	1.2	Unmethylated

Fig. 4 Analysis of the methylation status of the *MGMT* promoter, messenger RNA and protein expression of MGMT in GBM22-TMZ cell line treated with z-BP and temozolomide. We determined the methylation status of *MGMT* following treatment of the temozolomide-resistant GBM22-TMZ cell line with a combination of z-BP (50 μM, 100 μM, 200 μM, 400 μM and 600 μM) and 800 μM temozolomide.



0	25	50	100	200	400	800	μ M
1.0	1.0	1.0	1.0	1.1	1.1	1.1	MGMT
1.0	1.0	1.0	1.0	1.0	1.0	1.0	β-actin

0	25	50	100	200	400	800	1200	μ M
0.8	1.0	1.0	1.0	1.2	1.0	1.0	1.1	MGMT
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	β-actin

Fig. 5 E-BP does not alter MGMT expression in 8901 and GBM22-TMZ brain tumor cell lines. To test whether z-BP was the agent that induced MGMT silencing, 8901 GBM tumor cells were treated with e-BP (25 μM, 50 μM, 100 μM, 200 μM, 400 μM and 800 μM), and GBM22-TMZ brain tumor cells were treated with e-BP (25 μM, 50 μM, 100 μM, 200 μM, 400 μM, 800 μM and 1,200 μM).

resistance in patients with GBM [25-28]. Our previous study reported that combined treatment with z-BP and BCNU (bis-chloroethylnitrosourea) augments antineoplastic activity by z-BP-mediated MGMT promoter methylation in hepatocellular carcinoma [20]. In our current study of GBM cell lines, we found that higher MGMT expression in tumor cells resulted in higher IC_{50} values for temozolomide. Because we previously reported that z-BP is cytotoxic to malignant glioma owing to translocation of Nurr-77 from the nucleus to the cytoplasm [16], it was urgent to determine if the effect of temozolomide and z-BP against malignant glioma tumors was synergistic or additive.

In this study, we used two GBM cell lines to assess the relative effects of MGMT activity on the ability of

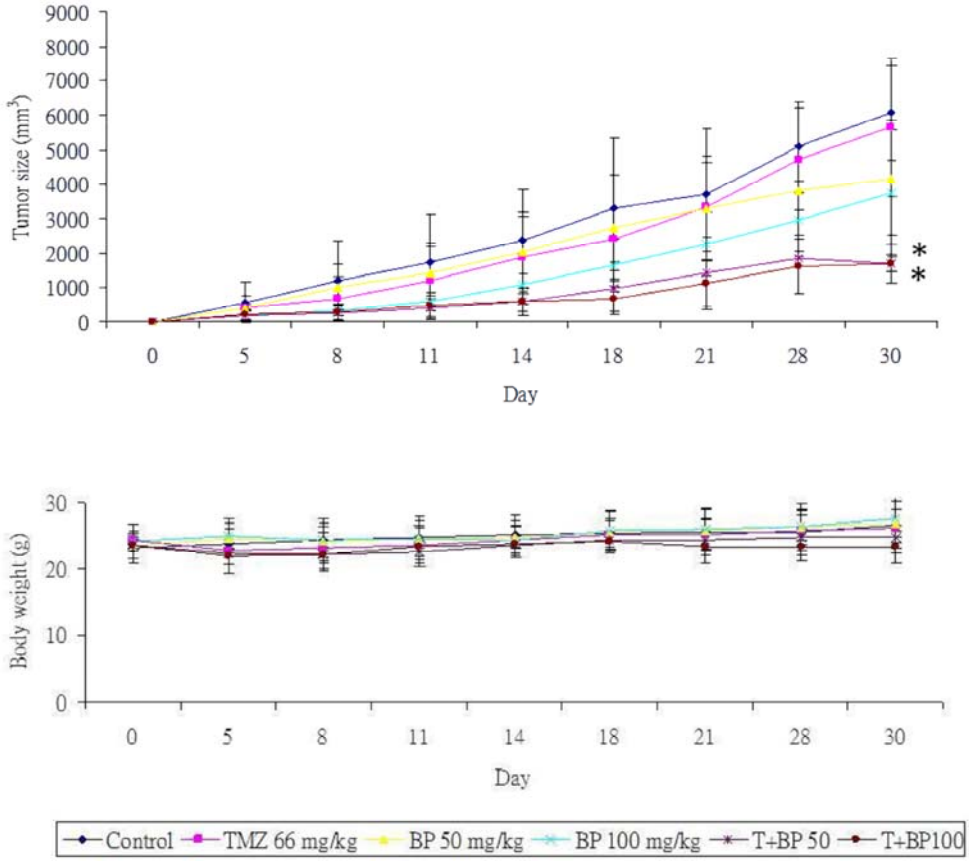


Fig. 6 Enhancement of the efficacy of temozolomide in the temozolomide-resistant GBM22-TMZ GBM cell line when combined with z-BP. Xenografts in BALB/c nude mice were performed by implanting approximately 2 × 10^6 tumor cells into the dorsal subcutaneous tissue. When tumor volumes reached 100-250 mm^3, tumor-bearing mice were subcutaneously given vehicle control, 66 mg/kg temozolomide alone, 50 mg/kg z-BP alone, 100 mg/kg z-BP alone or 50 mg/kg or 100 mg/kg z-BP combined with 66 mg/kg temozolomide on days 0-4 for 5 days. The panel shows the relative tumor volume of GBM22-TMZ cells in control and treated groups. The data represent the mean ± SD from six independent experiments. * Significantly different from control group, $p < 0.05$.

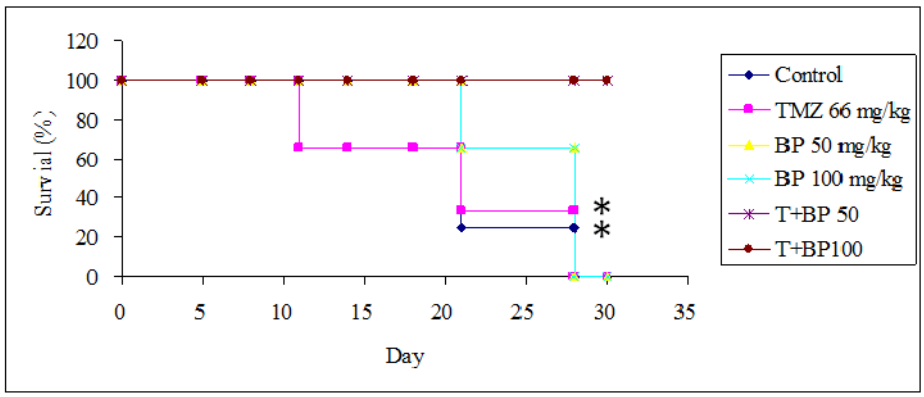


Fig. 7 Survival analysis of xenografted mice treated with a combination of z-BP and temozolomide. Animal mode was established like Fig 6. Survival was monitored daily. The mice were sacrificed when tumor volume exceeded 1,000 $mm^3$and that day was used as the final survival day. Results are shown as Kaplan-Meier survival curves. * Significantly different from control group, $p < 0.05$.

z-BP to potentiate the antitumor effects of temozolomide. First, our data indicate that z-BP was most effective in potentiating temozolomide in 8901 cell line, which have the highest MGMT activity of the cells we tested. Because z-BP alone inhibited tumor growth, we looked for a synergistic effect. CalcuSyn software was used to draw a dose-response curve for drug dosages, and the CI was calculated to determine the effect of drug interaction belong to synergism effect, additivity, or antagonism. For CI < 1, z-BP and temozolomide are synergistic. In this research, the combination of 50μM z-BP (IC_{50} for 8901 cell line: 218.62 μM) and 200 μM temozolomide (IC_{50} for 8901 cell line: 1201.6 μM) showed a CI of 0.841, indicating synergism. Further, the combination of 100 μM z-BP and 200 μM temozolomide showed a CI of 0.610 (Table 3). This dose-dependent relationship implies that the synergistic effect is due to z-BP and also suggests that the use of systemic z-BP (50-100 μM) plus temozolomide may enhance the sensitivity of malignant gliomas by about six fold.

The O^6-BG (O^6-Benzylguanine) mediated O^6-alkylguanine-DNA alkyltransferase depletion to augment the antineoplastic activity of BCNU agents has been reported before [29]. However, the use of O^6-BG plus a smaller dose of BCNU was not effective against BCNU-resistant GBM [30]. Therefore, we tested whether z-BP plus temozolomide could restore sensitivity to temozolomide-resistant malignant gliomas. In the *in vitro* study, we found that combination therapy with 50 μM z-BP (IC_{50} for GBM22 cell line: 450.9 μM) enhanced the sensitivity (IC_{50}) to temozolomide from >3,200 μM to 800 μM (Table 4, CI = 0.894). Thus, the dose of temozolomide could be reduced by 75% when given with z-BP. The dramatic decrease in the amount of temozolomide required may decrease the incidence of or delay in the time to temozolomide resistance. An *in vivo* study that examined tumor reduction and that generated a survival curve showed that 66 mg/kg temozolomide cannot be used to treat the temozolomide-resistant cell line GBM22-TMZ [31]. However, we observed that a combination of 50-100 mg/kg z-BP and 66 mg/kg temozolomide significantly inhibited tumor volume compared to either z-BP or temozolomide alone ($p < 0.01$, Figs. 6 and 7). Moreover, the survival curve demonstrated 100% survival with a combination of z-BP and temozolomide and no survival with temozolomide alone at 28 d (Fig. 7). In summary, we conclude that z-BP is effective in potentiating the effects of temozolomide in temozolomide-resistant cells and that z-BP restores sensitivity of cells to temozolomide.

The concomitant use of z-BP and temozolomide markedly enhanced antitumor activity. Temozolomide resistance results from at least three mechanisms including: (a) increased activity of the DNA repair protein MGMT, which removes O6-methyl adducts from the O6 position of guanine in DNA; (b) deficiencies in the DNA mismatch repair system, resulting in microsatellite instability and tolerance of O6-methylguanine adduct DNA mismatches [32]; (c) in addition, because temozolomide also generates N-methylated bases (N3 and N7), which can be removed by the BER (base excision repair) system [33], robust BER activity can result in temozolomide resistance[33]. The abundant nuclear enzyme PARP (poly(ADP-ribose) polymerase) senses both single- and double-stranded DNA breaks and initiates DNA repair; PARP is central to BER and the removal of methylated N3 and N7 adducts. PARP inhibitors (e.g., ABT-888) may overcome temozolomide resistance [32]. Our current studies focused on exploiting z-BP-mediated downregulation of MGMT. Our results demonstrated that the addition of 50-100μM z-BP to temozolomide restored sensitivity to temozolomide-resistant malignant glioma cells. This amount of z-BP is enough to increase MGMT promoter methylation and downregulate MGMT protein level (Figs. 1 and 3). The methylation capability induced by z-BP is not different between

z-BP alone and the z-BP/temozolomidecombination (Figs. 1 and 2). In addition, 50 μM and 100 μM z-BP had a similar effect on MGMT promoter methylation in the temozolomide-resistant GBM22-TMZ cell line compared to the sensitive GBM 8901 cell line (Figs. 1 and 3). The effective sensitization to temozolomide by ABT-888 is lost with the development of temozolomide resistance (GBM22-TMZ) in glioblastoma xenograft lines [32]. As mentioned above, resistance to temozolomide therapy requires integrity of the MGMT repair protein, a deficient mismatch repair system, and decreased activity of the BER system. Escape from either pathway leads to significantly increased cell killing by temozolomide. Therefore, in conjunction with the lack of temozolomide sensitization by ABT-888, the authors suggested that resistance may be caused by incomplete disruption of BER in these tumor lines by PARP inhibition. Our results show that z-BP can sensitize temozolomide-resistant GBM22-TMZ cell line *in vitro* and *in vivo*. Therefore, it was reasonable to speculate that z-BP can sensitize GBM-TMZ not only methylation MGMT promoter but also decreased other DNA repair mechanism. Thus, future studies should address the important roles of the BER and mismatch repair systems in mediating sensitization to temozolomide by z-BP.

As mentioned above, 50-100 μg/mL z-BP facilitated MGMT promoter methylation. This methylation capacity of z-BP was almost the same in the two cell lines 8901 and GBM22-TMZ, suggesting that the methylation mediated by z-BP does not affect temozolomide resistance. Interestingly, only z-BP but not e-BP altered the methylation status of MGMT (Fig. 5), suggesting an epigenetic mechanism. DNMT mammalian DNA methyltransferase) acts upstream of MGMT [34, 35].

Whether z-BP or e-BP affects DNMT activity requires further investigation. Further, Bobustuc et al. reported that anti anticonvulsant drug levetiracetam decreases MGMT protein and mRNA expression levels [36]. Chromatin immunoprecipitation analysis revealed that levetiracetam enhances p53 binding to the MGMT promoter by recruiting the mSin3A/ HDAC1 (histone deacetylase 1) corepressor complex. However, levetiracetam does not inhibit MGMT when the expression of p53, mSin3A, or HDAC1 is abrogated. Therefore, p53-induced inhibition of MGMT transcription requires simultaneous participation of both elements of the mSin3A/HDAC1 corepressor complex, which, in turn, is recruited by levetiracetam [36]. Interesting, in our preliminary chromatin immunoprecipitation study, z-BP but not e-BP increased p53 binding to the MGMT promoter, but z-BP did not affect MGMT expression in the p53-deficient cell line RG2 (data not shown). Whether z-BP activity is mediated by p53 and the mSin3A/HDAC1 corepressor complex requires further investigation.

5. Conclusions

In conclusion, (Z)-butylidenephthalide enhanced MGMT promoter methylation and decreased MGMT expression in a dose-dependent manner. By using TMZ resistant cell line GBM22-TMZ for vivo study, we demonstrated (Z)-butylidenephthalide combined with temozolomide showed significant antitumor effects and prolonged survival time. The combination of z-BP and temozolomide application implicates potential clinical for treating temozolomide-resistant GBM.

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