Local interstitial delivery of z-butylidenephthalide by polymer wafers against malignant human gliomas

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We have shown that the natural compound z-butylidenephthalide (Bdph), isolated from the chloroform extract of Angelica sinensis, has antitumor effects. Because of the limitation of the blood-brain barrier, the Bdph dosage required for treatment of glioma is relatively high. To solve this problem, we developed a local-release system with Bdph incorporated into a biodegradable poly-anhydride material, p(CPP-SA; Bdph-Wafer), and investigated its antitumor effects. On the basis of in vitro release kinetics, we demonstrated that the Bdph-Wafer released 50% of the available Bdph by the sixth day, and the release reached a plateau phase (90% of Bdph) by the 30th day. To investigate the in situ antitumor effects of the Bdph-Wafer on glioblastoma multiforme (GBM), we used 2 xenograft animal models—F344 rats (for rat GBM) and nude mice (for human GBM)—which were injected with RG2 and DBTRG-05MG cells, respectively, for tumor formation and subsequently treated subcutaneously with Bdph-Wafers. We observed a significant inhibitory effect on tumor growth, with no significant adverse effects on the rodents. Moreover, we demonstrated that the antitumor effect of Bdph on RG2 cells was via the PKC pathway, which upregulated Nurr77 and promoted its translocation from the nucleus to the cytoplasm. Finally, to study the effect of the interstitial administration of Bdph in cranial brain tumor, Bdph-Wafers were surgically placed in FGF-SV40 transgenic mice. Our Bdph-Wafer significantly reduced tumor size in a dose-dependent manner. In summary, our study showed that p(CPP-SA) containing Bdph delivered a sufficient concentration of Bdph to the tumor site and effectively inhibited the tumor growth in the glioma.

Keywords: carmustine (BCNU), glioblastoma multiforme (GBM), p(CPP-SA) wafer, z-butylidenephthalide (Bdph).

Brain tumors occur frequently in humans, with ~2000 new cases of primary central nervous system tumors diagnosed annually in Taiwan.
alone. Although brain tumors constitute only ~1% of all cancers, they are responsible for 2.5% of the overall cancer-related mortality.\(^1,^2\) The incidence of malignant childhood brain tumors is secondary only to leukemia. Glioblastoma multiforme (GBM) and malignant gliomas are highly vascularized and invasive neoplasms. The diffusely invasive properties of malignant gliomas make them nearly impossible to completely resect, making surgery, radiotherapy, and eventually chemotherapy the standard treatment.\(^3^-^6\) Despite this, the median duration of survival of patients with glioblastoma is only 30 weeks. The dismal prognosis of malignant gliomas such as GBM warrants continued investigation of new therapeutic options, with an early introduction of promising agents into clinical trials. Chemotherapy is usually reserved for recurrent tumors that have already been treated with surgery and radiotherapy, for partially removed tumors, or for individuals for whom surgery was not feasible and the effects of radiotherapy were limited.\(^7\) Various chemotherapy schemes are commonly used, with most consisting of the administration of high doses of drugs. Current chemotherapy regimens are limited for several reasons, including toxicity, development of drug resistance, limited success in overcoming the blood-brain barrier (BBB), and limited therapeutic effects.\(^8^-^12\)

Six major compounds have been isolated from *Angelica sinensis*: (E)-ligustilide, (Z)-ligustilide, z-butylidenephthalide, palmitic acid, \(\beta\)-sitosterol, and ferulic acid. z-butylidenephthalide (Bdph or K1; molecular weight, ~188.22) and ligustilide (K2; molecular weight, ~190.23) are particularly abundant,\(^13\) with the former exhibiting more potency than ligustilide. In conscious rats, Bdph relieves angina without affecting blood pressure or the heart rate.\(^14,^15\) Previously, we demonstrated that the acetone extract of *A. sinensis* inhibits the proliferation of cancer cells in vitro\(^16\) and that the subsequently obtained chloroform extract of *A. sinensis* antagonizes brain tumor cells in vitro and in vivo.\(^17\) We examined the antitumor effects of Bdph on neuroblastoma, lung cancer, melanoma, teratoma, leukemia, breast cancer, and hepatocellular carcinoma in vitro and on GBM brain tumors both in vitro and in vivo. Compared with the clinical chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), Bdph inhibits the proliferation of tumor cells more effectively, including those from neuroblastoma, lung cancer, melanoma, teratoma, leukemia, breast cancer, and hepatocellular carcinoma.\(^18\) In addition, when GBM cells were treated with Bdp, significant inhibitory effects on proliferation and cell cycle progression were found, as was induction of apoptosis. Subsequently, in an in vivo study, mice harboring cells from the human GBM tumor DBTRG-05MG and the rat GBM tumor RG2 were injected subcutaneously or intracerebrally with Bdph. Tumor growth was inhibited, magnetic resonance imaging showed a reduction in tumor volume, and the survival rate increased.\(^17,^18\) Finally, Bdph up regulates the expression of cyclin kinase inhibitors, including p21 and p27, decreases the phosphorylation of Rb proteins, and downregulates the expression of cell-cycle regulators, resulting in cell-cycle arrest at the G0/G1 phase.\(^17,^18\) These in vitro and in vivo anticancer effects indicate that Bdph may function as a new anti–brain tumor drug.

To identify the genes involved in Bdph-induced growth arrest and apoptosis, we used an oligodeoxynucleotide-based microarray technique to screen for genes upregulated by Bdph. Among these genes, we found that members of the nuclear receptor Nur77 superfamily (NR4A1, NR4A2, and NR4A3) were upregulated immediately after Bdph treatment.\(^19\) NOR-1 (NR4A3), Nurr1 (NR4A2), and Nur77 (NR4A1) are immediate early genes induced by serum, growth factors, receptor binding, and apoptotic stimuli.\(^20^-^23\) These proteins share similar structural features,\(^24\) but their physiological ligands have not been identified, making them orphan receptors.\(^25\) NOR-1, Nurr1, and Nur77 have previously been implicated in cell growth and/or survival and apoptosis.\(^24\) Nur77-mediated apoptosis has been extensively studied in T cells and several cancer cell lines.\(^21,^23,^26^-^28\) Two Nur77-mediated apoptosis mechanisms have been reported. As a transcription factor, Nur77 appears to up-regulate genes that promote apoptosis (eg, Fas ligand, tumor necrosis factor–related apoptosis-inducing ligand, and Nur77 downstream gene–1 and –2).\(^29^-^31\) Nur77 also translocates to mitochondria, where it interacts with Bcl-2 to form a pro-apoptotic complex in response to apoptotic stimuli. This interaction reverses the function of Bcl-2 from anti-apoptotic to pro-apoptotic, triggering cytochrome c release and apoptosis, as demonstrated in LNcaP human prostate and other cancer cells.\(^23,^28\) In our previous studies of Bdph,\(^16^-^19,^32\) in vitro and in vivo anticancer effects suggested that Bdph might serve as a new drug against human brain tumors. Systemic administration of Bdph for the treatment of brain tumors would, however, require very high doses to achieve penetration of the BBB—an approach likely to generate severe toxicity.

Local delivery of drugs using controlled-release polymers is a safe alternative for delivering chemotherapy agents to malignant brain tumors. Controlled-release polymers bypass the BBB, preventing systemic toxicity.\(^35\) One such therapy, Gliadel (Guilford Pharmaceuticals Inc.) has received regulatory approval for both recurrent and newly diagnosed malignant gliomas. This treatment involves local delivery of carmustine using biodegradable polymers and prolongs survival of patients with malignant gliomas, although only by \(~2\) months.\(^33\) Adverse effects include higher incidences of wound infection and dehiscence.\(^33^-^35\) Therefore, a safer and more effective controlled-release wafer is needed.

In this study, we tested the cytotoxic activity of controlled release of Bdph from p(CPP-SA) wafers on the malignant glioma cell lines DBTRG and 8401 in vitro and in vivo. We evaluated the safety and efficacy of Bdph-Wafers that were subcutaneously implanted in the flanks of animals that received xenografts of DBTRG human malignant glioma cells. Finally, using spontaneous brain tumors generated by transgenic FGF-SV40 mice, we analyzed whether implanted wafers can deliver Bdph into the brain. Bdph-Wafers
not only decreased the size of tumors but also kept its concentration for 30 days. Thus far, there has been no brain edema, no delay in wound healing, no CSF leakage, and no brain infection observed. Here, we propose an alternative wafer-based compound for the treatment of human brain tumors.

Materials and Methods

Chemicals

Bdph (A10353) was purchased from Alfa Aesar. Bdph was dissolved in dimethylsulfoxide (DMSO; Sigma), incubated with shaking at 25°C for 1 h, and stored at 4°C until use. BCNU, sebacic acid (SA), and 1,3-bis (4-carboxyphenoxy) propane (CPP) were obtained from Sigma-Aldrich.

Cell Lines and Cell Culture

DBTRG-05MG human GBM cells and RG2 rat GBM cells were obtained from the American Type Culture Collection. G5T/VGH human GBM cells and human GBM 8401 cells were obtained from Bioresources Collection and Research Center. DBTRG-05MG and human GBM 8401 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum, and G5T/VGH and RG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Analysis of Cell Cytotoxicity

Cell viability was evaluated using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. In brief, cells were incubated in eight 96-well plates (5 × 10³ cells/well) containing 100 μL of growth medium. Cells were grown for 24 h and then treated with 100 μL of Bdph (0–250 μg/mL) dissolved in medium. The DMSO concentration in each preparation was ≤0.02%. After 24, 48, or 72 h of incubation, the drug-containing medium was replaced with 50 μL of fresh medium containing MTT (400 μg/mL) for 6–8 h. The MTT-containing medium was then removed, and 100 μL of DMSO was added to each well. Absorbance (550 nm) of the solutions was detected using an MRX Microtiter Plate Luminometer (Dynex Technologies). The absorbance of untreated cells was set at 100%. The half-maximal inhibitory concentration was defined as the concentration that caused a 50% decrease in the absorbance of the drug-treated cells, compared with the untreated cells.

Polymer Preparation

SA Prepolymer Preparation—SA was recrystallized 2 times in alcohol. SA monomers (2.7 g) were refluxed with 60 mL excess acetic anhydride for 30 min at 135°C–140°C in a vacuum (10⁻⁴ torr). Excess unreacted acetic anhydride was removed, and the SA prepolymer was dried by evaporation under vacuum at 60°C and then dissolved in dried toluene. The SA prepolymer was precipitated in a 1:1 (v/v) mixture of dry ethyl ether and dry petroleum ether from dried toluene (10:1, v/v) overnight. Excess ethyl ether and petroleum ether were removed, and the SA prepolymer was dried in a vacuum.

CPP Prepolymer Preparation—CPP monomers (3 g) were refluxed with 50 mL excess acetic anhydride for 30 min at 150°C in a vacuum (10⁻⁴ torr). After cooling, the solution was filtered with filter papers. Excess unreacted acetic anhydride was removed, and the CPP prepolymer was recrystallized at 0°C. The remaining unreacted acetic anhydride was removed, and dry ether was added to wash the CPP prepolymer overnight. The dry ether was removed, and the CPP was dried in a vacuum. The CPP prepolymer was washed with dimethylformamide (DMF). Dry ether (DMF/dry ether, 1:9) was then added and incubated overnight. The dry ether and DMF were removed, and the CPP prepolymer crystals were dried in a vacuum.

Poly(CPP-SA) Copolymer Preparation—A mixture of CPP prepolymer and SA prepolymer at a ratio of 20:80 was added to glass tubes (2 × 20 cm) in a vacuum. The CPP and SA prepolymers were heated at 180°C in an oil bath for 1 min, and the pressure was reduced to 10⁻⁴ torr. Throughout the polymerization, the vacuum pressure was reduced every 15 min. After 1.5 h, the poly(CPP-SA) copolymers were washed with dichloromethane, and then petroleum ether was added to precipitate the poly(CPP-SA) copolymers. Finally, the poly(CPP-SA) copolymers were washed with anhydrous ether and dried in a vacuum. p(CPP-SA)(20:80) polymers containing Bdph or BCNU were synthesized according to the method of Domb and Langer. Bdph was combined with p(CPP-SA; 20:80) polymers containing Bdph or BCNU and then dried in a vacuum. p(CPP-SA)(20:80) containing 5% BCNU mixture was also prepared in a similar manner. Adding methylene chloride dissolved the mixture and yielded a 10% solution (w/v). Desiccating the solution in a vacuum for 72 h yielded a dry powder. Bdph-Wafers (final weight, 100 mg) were prepared by compression molding of the Bdph polymer powder with a stainless steel mold (internal diameter, 13 mm) under light pressure from a Carver Press at 361 944 torr. Wafers containing 3% BCNU were prepared by codissolving BCNU and polymer in methylene chloride and then drying and forming the mixture as described above.

Release Kinetics

To measure the release of Bdph, Bdph-Wafers were placed into a scintillation vial with 1.0 mL of 0.1 M phosphate-buffered saline (pH 7.4) containing 1 mL n-octanol, and the mixture was incubated at 37°C. Harn et al.: α-Butylidenephthalide wafer to treat human glioblastoma

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The solution was removed at various time points, and fresh buffer was added. The amount of Bdph released into the buffer was measured with a spectrophotometer.\(^4^0\) Bdph was absorbed at a wavelength of 310 nm; therefore, we measured the absorption of methanol at 310 nm as background to determine the concentration of Bdph.

**Xenograft Animal Studies**

Male F344 rats (230–260 g) and male Foxn1 nu/nu mice (age, 10–12 weeks) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). All procedures were performed in compliance with the standard operating procedures of the Laboratory Animal Center of National Dong Hwa University (Hualien, Taiwan) and China Medical University Hospital (Taichung, Taiwan). Syngeneic F344 rats (6 per group) were given subcutaneous back implants of 1 \times 10^6 RG2 cells. Five days after RG2 cell implantation, animals in each group were treated with a subcutaneous implant of 3% or 10% Bdph-Wafer, 3% BCNU-Wafer, or wafer alone under the original cell injection site.

In addition, nude mice (5 or 6 per group) also received subcutaneous implants of 1 \times 10^6 DBTRG-05MG cells and subcutaneous implants of 3% or 10% Bdph-Wafer, 3% BCNU-Wafer, or wafer alone under the original injection site after the tumor cell implantation. Tumor sizes were measured using calipers, and the volume was calculated as length \times width^2 \times \pi \times 0.5236.\(^4^1\) Animals were killed when the tumor volume exceeded 25 cm^3 in rats and 1000 mm^3 in mice. The day of sacrifice constituted the final survival day for the animals.

**Animal Studies of Spontaneous Brain Tumors**

FGF-SV40 transgenic mice were a gift from Professor Ing-Ming Chiu and Dr. Adrienne Frostholm (Ohio State University; Columbus, Ohio).\(^4^2\) All procedures for the animal studies were approved by the Institutional Animal Care and Use Committee of China-Medical University. Eighteen 30-day-old FGF-SV40 transgenic mice were randomly divided into 3 groups, as follows: the control wafer group, the 10% Bdph-Wafer group, and the 15% Bdph-Wafer group \((n = 6 \text{ in each group}). Before the wafer implant, mice were anesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate. After anesthesia, the mice had their heads shaved and were prepared aseptically with a 70% ethanol and povidone-iodine solution. To put the control wafer, 10% Bdph-Wafer, or 15% Bdph-Wafer in place on the cerebellum, we removed the occipital bone with a drill and forceps while using an operating microscope to observe the surgical site. A 5-mm hole was made in the skull with a trephine in an area posterior to the coronal suture and lateral to the sagittal suture (2 mm from each suture). An incision (4-mm deep) was made in the exposed dura with a 11 scalpel blade. Blood was removed using sterile cotton-tipped swabs, and a wafer was inserted in the cortical defect at a depth of 3 mm and was placed on the cerebellum. After ensuring homeostasis, the skin was sutured with surgical clips. The mice were placed on a warming pad until they awakened.

**Animal Sacrifice andHistological Evaluation**

Thirty days after implantation, FGF-SV40 transgenic mice were sacrificed with use of an intraperitoneal injection of 800 mg/kg chloral hydrate. The skull was opened, and the brain was removed. The brain was fixed in 10% formalin, and each brain was cut into 11 (1-mm-thick coronal) sections. Sections were then embedded in paraflin. The resulting paraflin blocks were sectioned with a microtome to produce 8-μm cross-sections, which were stained with hematoxylin and eosin (H&E) and were stained immunohistochemically for glial fibrillary acidic protein (GFAP). The tumor volume was determined by measurement of the length and width of the tumor. The tumor volume at day \(n\) \((TVn)\) was calculated as \(TV = (length \times width)^2/2\). The relative tumor volume at day \(n\) \((RTVn)\) versus day 0 was expressed according to the following formula: \(RTVn = TVn/TV0\). Tumor regression \([T/C (\%)]\) in treated versus control mice was calculated using \(T/C (\%) = (\text{mean RTV of treated group)/(mean RTV of control group}) \times 100.\) We calculated the tumor volume on the basis of the area of each section and the total coronal section thickness (1 mm) using a microscopy system (Olympus IX70).

**Statistics**

Data were expressed as the mean ± standard deviation. Statistical significance was analyzed using the Student test and Mantel-Cox test. Survival analysis was performed using the Kaplan-Meier method. A value of \(P < .05\) was considered statistically significant.

**Results**

**Polyanhydride Wafer Preparation**

CPP-SA copolymers were synthesized by thermal melt polycondensation of CPP and SA in excess acetic anhydride. CPP-SA copolymers were identified with IR and \(^1\)H NMR. Using IR spectroscopy of CPP-SA copolymers, the characteristic signal of an anhydride bond was observed at 1812.76 cm\(^{-1}\). Using \(^1\)H NMR spectroscopy of CPP-SA copolymers, the characteristic signals of the aromatic protons in CPP were observed at 6.9–8.2 ppm (Fig. 1A). The characteristic signal of the methylene protons in SA was measured at 1.3 ppm. The ratio of CPP to SA in the copolymers ranged from 1:4 to 1:5 according to the characteristic peak. The intensity of CPP and SA in Fourier transform infrared spectroscopy of the CPP-SA copolymer is shown in Figure. 1B.
Controlled Release of Bdph-Wafer and Its Cytotoxic Effects in Vitro

The release kinetics of 3% BCNU-Wafers and 3%, 10%, and 15% Bdph-Wafers were measured over 30 days in vitro (Fig. 2). Sustained release of Bdph and BCNU was observed in all wafer formulations. 10% Bdph-Wafer released 50% of the available Bdph by the sixth day and reached a plateau phase (90% of Bdph released) at the 30th day (Fig. 2). p(CPP-SA) with 10% Bdph inhibited growth of malignant glioma RG2 cells by 90%, compared with control wafers (Fig. 3A). In addition, the morphology of GBM tumor cells gradually changed, and cells detached from the bottom of the culture plate after treatment with 10% Bdph-Wafers (Fig. 3B). In contrast to the untreated cells, most detached GBM cells showed shrinkage, nuclear fragmentation and chromatin condensation after treatment with 10% Bdph-Wafers (Fig. 3B). We also observed many apoptotic bodies after treatment with 10% Bdph-Wafers, as well as detached GBM cells that were undergoing apoptosis (data not shown).

Apoptotic Pathways and Nur77 Translocation Induced by Bdph-Wafers

To confirm the results of our previous oligodeoxynucleotide-based microarray analysis, we examined the expression of the orphan receptor Nur77 in Bdph-treated RG2 cells using reverse-transcription polymerase chain reaction. After treatment with Bdph...
for various times, mRNA expression of Nurr77 was induced in the RG2 cell line in a time-dependent manner. Nurr77 mRNA was significantly induced 30 min after Bdph treatment, and the expression was maintained 6 h after treatment (Fig. 4A). Nur77 migrates from the nucleus to the mitochondria and induces apoptosis in response to certain apoptotic stimuli.23 To examine whether translocation of Nur77 occurred in response to Bdph, the localization of Nur77 was examined using a Nur77-specific antibody, and localization was observed with a fluorescence microscope (Fig. 4B and 4C). Immunoblotting showed that Nur77 was predominantly localized in the nuclear fraction in the absence of Bdph treatment (Fig. 4B). Nur77 was more abundantly expressed in the nucleus than in the cytoplasm. After treatment with Bdph for 24 h, Nur77 translocated from the nucleus to the cytoplasm (Fig. 4B). To further confirm this observation, cytosolic and nuclear fractions were evaluated using Western blot.
Finally, we used Western blot analysis to determine the signaling pathway involved in Bdph-induced Nur77 gene expression. PKC, JNK, ERK, and AKT were phosphorylated after Bdph treatment for 1 h (Fig. 5A). To determine which pathway played a role in Bdph-induced growth inhibition of
RG2 cells, these cells were treated with Bdph in the presence or absence of the PKC inhibitor Go6983 (0.5–1 μmol/L), JNK inhibitor SP600125 (5–10 μmol/L), mitogen-activated protein kinase kinase 1/2 inhibitor PD98059 (5–10 μmol/L), or PI3K/AKT/GSK3β inhibitor wortmannin (20–40 μmol/L). The MTT assay showed that RG2 cell viability increased when cells were pretreated with the PKC inhibitor Go6983 at 1 μM and with Bdph (Fig. 3B). PKCβ signaling pathways involved in the Bdph-induced Nur77 migration has also been identified (Fig. 5C–E).

**In Vivo Therapeutic Effects of Bdph-Wafers on Animals with Subcutaneous GBM Tumors**

To investigate the in situ antitumor effects of Bdph-Wafers on rat GBM cells, F344 rats were given 1 × 10⁶ RG2 cells and treated subcutaneously with 3% or 10% Bdph-Wafers, 3% BCNU-Wafers, or polymer alone. We observed a significant inhibitory effect on tumor growth with the 3% and 10% Bdph-Wafers and with the 3% BCNU-Wafers, compared with either polymer or RG2 cells alone (P < .005) (Fig. 6A). The mean tumor size at day 30 was 2070.79 ± 784.90 mm³ in the RG2 group, 1586.30 ± 243.69 mm³ in the polymer alone group (no drug), 346.71 ± 521.68 mm³ in the 3% Bdph-Wafer group, 87.89 ± 167.44 mm³ in the 10% Bdph-Wafer group, and 357.48 ± 27.30 mm³ in the 3% BCNU-Wafer group. In addition, immunohistochemical staining for Ki-67, which indicates cell proliferation, demonstrated a substantial decrease in cell proliferation in the rats treated with 10% Bdph-Wafers (Fig. 6C). Immunohistochemical staining for caspase-3, which indicates cell apoptosis, showed a substantial increase in the 10% Bdph-Wafer group (Fig. 6C). Finally, no drug-related toxicity was observed in animals given 10% Bdph-Wafers, as evaluated by measurements of body weight (Fig. 6B) and histological analyses of various organs (data not shown). We did, however, observe significant weight loss in animals treated with 3% BCNU-Wafers (Fig. 6B).

**Therapeutic Effects of Bdph-Wafers on Xenograft Tumor Growth**

To determine whether Bdph-Wafers suppress human GBM tumor growth, nude mice were inoculated with 1 × 10⁶ human DBTRG-05MG cells and implanted with 3% and 10% Bdph-Wafers or with control wafers on day 5. The 3% and 10% Bdph-Wafers inhibited tumor growth (Fig. 7). Significant suppression of tumor growth in the 3% and 10% Bdph-Wafer groups was observed (Fig. 7A). The mean values for tumor size at day 39 were 1098.46 ± 170.11 mm³ in the control group, 627.71 ± 137.44 mm³ in the 3% Bdph-Wafer group, and 304.4 ± 150.79 mm³ in the 10% Bdph-Wafer group (P < .01, for control versus 3% and 10% Bdph-Wafers) (Fig. 7A).

**Therapeutic Effects of Bdph-Wafers on Spontaneous Brain Tumors in Mice**

To verify their antitumor activity in spontaneous brain tumors, Bdph-Wafers were implanted in 30-day-old FGF-SV40 transgenic mice. Mice were killed 20 days after wafer implantation, and coronal sections from each mouse brain were stained with H&E. The mean tumor volume 20 days after the wafer was implanted was 0.33 ± 0.33 mm³ for the control group, compared with 0.16 ± 0.22 mm³ for the 10% Bdph-Wafer group and 0.12 ± 0.02 mm³ for the 15% Bdph-Wafer group. Furthermore, the relative tumor volume was reduced by 51% in the 10% Bdph-Wafer group and by 64% in the 15% Bdph-Wafer group (Fig. 8A). There was a significant inhibitory effect on tumor growth for both the 10% and 15% Bdph-Wafer groups versus the control (wafer-only) group (P < .05) (Fig. 8B). As a marker for astrocyte tumor cells, GFAP staining decreased remarkably after a 20-day exposure to the Bdph-Wafers as compared with the control group (Fig. 8C). In the 15% Bdph-Wafer group, tumor size, as indicated by GFAP-positive cells, was significantly reduced, and the cells were localized to a smaller region, whereas in the control group, GFAP-positive cells were more widespread and had even invaded the fourth ventricle (Fig. 8C). Finally, tumor volume was reduced by the Bdph-Wafers in a dose-dependent manner (Fig. 8A). FGF-SV40 transgenic mice survival was significantly prolonged in the Bdph-Wafer group in comparison to the control group (Fig. 8D; P < .01).

**Discussion**

Telomerase is widely expressed in 80%–90% of human cancers, but it is almost undetectable in normal somatic cells. In human brain tissue, telomerase activity is observed in 89% of glioblastomas and 45% of anaplastic astrocytomas, but it is absent in normal brain tissue. 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). This suggests that telomerase activation is involved in the establishment of cellular immortality and may therefore be a critical step in carcinogenesis. Therefore, inhibiting telomerase activity may be a therapeutic strategy for selectively targeting malignant gliomas and sparing normal brain tissue.

We examined the naturally occurring compound Bdph to investigate its possible antitumor activity in vitro and in vivo. We treated human glioblastoma cells with Bdph and observed a dose-dependent decrease in human telomerase reverse-transcriptase mRNA expression and a concomitant increase in p16 and p21 expression (data not shown). This was supported by data from a mouse xenograft model in which Bdph suppressed telomerase activity and inhibited tumor proliferation, which resulted in tumor senescence (data not shown).
Although telomerase is absent from most human somatic tissue, telomerase is expressed in adult germline tissues and in bone marrow hematopoietic cells. The presence of this telomerase activity may result in unwanted adverse effects if Bdph is systemically administered to treat malignant gliomas; this may pose a particular problem for long-term treatment regimens. In an attempt to solve this potential issue, we tested the local, controlled release of drugs bound to polymers. Overall, targeting the telomerase gene using a local delivery system to produce antitumor effects with drugs such as Bdph may result in a clinically applicable therapy in the future.

Fig. 6. Bdph-Wafers inhibited tumor growth in a syngeneic rat glioblastoma multiforme (GBM) model. RG2 cells (1 × 10⁶) were implanted subcutaneously into the hind-flank region of F344 rats. Five days after RG2 cell transplantation, the rats were treated with wafers alone, 3% Bdph-Wafers, 10% Bdph-Wafers, or 3% BCNU-Wafers. The resulting tumor sizes (A) and rat body weights (B) are shown as the mean ± standard deviation *P < .05. (C) Immunohistochemical staining was performed on GBM tissues (at day 30 after implantation of RG2 cells). Sections of GBM tumors from animals receiving wafers only, 3% Bdph-Wafers, 10% Bdph-Wafers, and 3% BCNU-Wafers were immunohistochemically stained for Ki-67, cleaved caspase-3, or Nur77. Positive cells are stained brown (original magnification, ×400; bar = 50 μm).
tumor and normal cells. As a consequence, many adverse effects are observed even with local application. Adverse reactions are both local and mild (e.g., nausea and vomiting) and systemic and aggravating (e.g., infection, pulmonary embolus, and hemorrhaging). In contrast, systemic administration of Bdph results in only a mild hepatic impairment and slight hyperglycemia accompanying a 7.5 g/kg median lethal dose (LD$_{50}$). Their mild side effect is due to Bdph being a targeted drug. The effector of Bdph is the orphan receptor Nurr77, which translocates from the nucleus to the cytoplasm and leads to tumor apoptosis.$^{23,24}$ In our previous study, we showed that Bdph increases Nurr77 transcription via an AP-1 motif.$^{49,50}$ Nurr77 has been implicated in growth inhibition and apoptosis,$^{19,21,23,26-28,32}$ suggesting that Nurr77 induction may be an early event during Bdph-induced apoptosis in GBM cells. To determine whether the same molecular mechanism is involved in the antitumor effect, we examined the effects of Bdph on tumor viability (Fig. 3), signal transduction (Fig. 5), Nurr77 protein translocation (Fig. 4), and histochemical staining of in vivo brain tumor cell xenografts (Fig. 7) when it is released from biodegradable wafers. Our results implicated the PKC pathway in signal transduction (Fig. 5A), showed Nur77 translocation from the nucleus to the cytoplasm (Fig. 4), and demonstrated caspase expression leading to tumor cell apoptosis (Fig. 6C). These results are consistent with the effects of Bdph when it is not incorporated into polymer form. Our findings suggest that the Bdph-Wafer retains its natural biological antitumor function.

Sekiya et al. (2000) reported the biodistribution of Bdph. Less than 1 h after dermal application, labeled
Bdph (unchanged [8-14C]butylidenephthalide and/or its metabolite) was detected at the application site and in the liver, bile, and kidney. At 4 h, the labeled compound was still strongly detected in the intestines, although the total amount in the body decreased. The biodistribution data showed that only 0.029 μg/g of brain tissue was detected after 1 h. Thus, only 3% of the total amount can reach the brain. Although Bdph is a hydrophobic compound, the BBB most likely contributes to the low amount of Bdph found in the brain. Therefore, controlled
local delivery of the drug to the target could circumvent this problem. We showed that approximately one-half of the available Bdph in the 10% Bdph-Wafer (≏5 mg) was released from the degraded polymer at 6 days and that 99% was released by the 30th day. Thus, nearly 100% of the available Bdph reached the brain tumor using our biodegradable polymer.

Sekiya et al. (2000) also reported the pharmacokinetics of Bdph. After dermal application, the total radioactivity from labeled Bdph was decreased because of excretion into the urine. With intravenous administration, 80% of the administered Bdph was excreted into the urine within 24 h, whereas only 5% was excreted into the feces during this time. In contrast, detectable amounts of Bdph released from our local, controlled delivery system were maintained for up to 30 days (Fig. 2). Our results demonstrate that the Bdph-Wafer not only led to an increase in the local compound concentration (ie, 50-fold) but also produced a concentration that remained stable for up to 30 days.

Finally, compared with the carmustine-Gliadel wafer, the Bdph-Wafer targets telomerase and therefore is expected to have no significant toxicity on surrounding normal brain cells. In addition, Bdph has a higher lethal dose than does carmustine (7500 mg/kg vs. 83 mg/kg), which encourages us to test higher concentrations and different formulations (such as glue or paste) as well as other biodegradable polymers (such as poly(lactic-co-glycolic acid), chitosan, and hydrogel) to increase the stability and local Bdph concentration.

Finally, to study the effect of the interstitial administration of Bdph against cranial brain tumors, FGF-SV40 transgenic mice were used. Our implantable biodegradable anhydride significantly reduced tumor size in a dose-dependent manner (Fig. 8). There was no brain edema, no delay in wound healing, no CSF leakage, and no brain infection—all symptoms that have been observed for BCNU-Wafers. This may be due to the fact that Bdph is less toxic (LD50, 7.5 g/kg) than BCNU (LD50, 20 mg/kg). In addition, in the FGF-SV40 mouse model, spontaneous brain tumors are located in the frontal region of the cerebellum and posterior region of the fourth ventricle. Because our wafer was implanted at the back of the cerebellum, it would seem that the Bdph-Wafer can penetrate brain tissue to reach the tumor without injury to normal cells. As a result, we believe that the Bdph-Wafer would have an even greater effect on a tumor when it is in direct contact with the cavity wall after tumor removal. In addition, this treatment may be useful for GBMs that are present in the brainstem, a location that is difficult to reach surgically.

We have shown that the antitumor effect of Bdph is due to Nur77 translocation from the nucleus to the cytoplasm, leading to cytochrome c release and tumor apoptosis. According to a pharmacokinetic study, the half-life of Bdph is ≏1 day, with excretion from urine. In this wafer-based system, Bdph is slowly released for up to 30 days (Fig. 2). More importantly, at 30 days after implantation, Nur77 translocation and tumor cell apoptosis were still observed in our nude mouse model (Fig. 6C). Thus, our wafer device maintains the stability and effectiveness of the Bdph compound for 30 days.

In summary, our study showed that polymers containing Bdph, a novel potential Nur77-induced apoptosis drug, increase the local Bdph concentration and maintain its stability, leading to significant inhibition of tumor growth.

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**Conflict of interest statement.** None declared.

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