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Corresponding Author: Dr. Horng-Jyh Harn, M.D. & Ph.D.

Corresponding Author's Institution: China Medical University and Hospital

First Author: Horng-Jyh Harn, M.D. & Ph.D.

Order of Authors: Horng-Jyh Harn, M.D. & Ph.D.; Shinn-Zong Lin; Li-Fu Chang; Cyong-Yue Liu; Dean-Kuo Hsieh; Fu-Chen Liu; Dar-Fu Tai; Po-Cheng Lin; Po-Yen Liu; Tzyy-Wen Chiou

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Abstract: We have shown that the natural compound z-butylidenephthalide (Bdph), which is 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 polyanhydride material, p(CPP-SA) (Bdph-Wafer) and investigated its antitumor effects. Based on 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 thirtieth day. To investigate the in situ antitumor effects of the Bdph-Wafer on glioblastoma multiforme (GBM), we used two 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 against 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.

Suggested Reviewers: Kwok-Fai So
hrmaskf@hkucc.hku.hk
Member of the Chinese Academy of Sciences, Co-Chairman of the Board of Director of the China Spinal Cord Injury Network (www.hku.hk/hkuscif), and Co-Director of the State Key laboratory of Brain and Cognitive Sciences at HKU (www.hku.hk/fmri/). He received his Ph.D. degree from MIT. He is one of the pioneers in the field of axonal regeneration in visual system. He was the first to show lengthy regeneration of retinal ganglion cells in adult mammals with peripheral nerve graft. He is currently using multiple approaches to promote axonal regeneration in the optic nerve and spinal cord. His team

identifies neuroprotective and regenerative factors including: peptide nanofiber scaffold, trophic factors, herbal extracts, other small molecules, immune responses and environmental manipulation. He is the author and co-author of over 200+ publications.

Koji Abe
abekabek@cc.okayama-u.ac.jp
Dr. Koji Abe is currently Professor and Chairman of Neurology at Okayama University Medical School in Japan. He graduated Tohoku University School of Medicine (M.D.), and then got Ph.D. title from Tohoku University under direction of Professor Kyuya Kogure. Professor Koji Abe has been publishing more than 350 papers on cerebral blood flow and metabolism and neurodegenerative diseases. His research interests cover many important fields of neurology especially in the mechanism of ischemic brain damage, gene and stem cell therapy, neuroprotection, and neuroimaging. He is currently president of the International Society of Cerebral Blood Flow and Metabolism.

Wai-Kwan Yung
wyung@mdanderson.org

Wise Young
wisey@mac.com

Opposed Reviewers: Yi-Lin Chen
ylchen@niu.edu.tw
conflict of interest

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Signature of Coauthor Completing This Form: Tzyy-Wen Chiou Date: March 3, 2010

Printed Name of Coauthor Completing This Form: Tzyy-Wen Chiou

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*Cover Letter

中國醫藥大學附設醫院
China Medical University Hospital
2, Yu-De Road, Taichung Taiwan, R.O.C.

Dear editor,

Our manuscript entitled "Local interstitial delivery of ***z***-butylidenephthalide by polymer wafers against malignant human gliomas" is submitted to "***Neuro-Oncology***".

Malignant glioma is still a lethal disease despite the adaptation of various modern technologies. New drug development is urgent for dealing this untreatable disease. ***z***-butylidenephthalide, a natural compound identified from *Angelica sinensis*, is found to be very effective in causing the apoptosis of malignant glioma by the molecular mechanism of the translocation of Nur-77 to the mitochondria. In this paper, we further incorporate Bdph into polymer wafers for local interstitial therapy. This Bdph-Wafers were tested in three different kinds of malignant glioma animal models including in a transgenic mice, in which malignant glioma occurred spontaneously in the brain stem at age of 4 weeks. Although the Bdph-Wafers were put on the surface of cerebellum (for away from the glioma in the brain stem), the gliomas were well-controlled through the molecular mechanism of Nur-77.

We are very excited about this development for treating the malignant glioma in the near future.

In here, we suggest the four persons as a reviewer for our manuscript. Each of them is outstanding in **malignant glioma research** and they are the pioneers in **brain tumor therapy**. This is the reason we suggest they can review our work and give us advantageous suggestion for future work.

The authors have no conflicts of interest to declare.
Sincerely yours,

Horng-Jyh Harn , M.D., Ph.D.
Director, Department of Pathology
Professor of Pathology
China Medical University & Hospital
No. 2, Yu de Rd., North District, Taichung 40447, Taiwan,
Phone: 886- 4-22052121 ext.2668
Fax: 886-4-8564656
E-mail: duke_harn@yahoo.com.tw

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

Horng-Jyh Harn,[1#] Shinn-Zong Lin,[2#] Li-Fu Chang,[3] Cyong-Yue Liu,[3] Dean-Kuo Hsieh,[4] Fu-Chen Liu,[5] Dar-Fu Tai,[5] Po-Cheng Lin,[3] Po-Yen Liu,[6] Tzyy-Wen Chiou[3,*]

[1]Pathology Department, China Medical University & Hospital, Taichung, Taiwan, ROC

[2]Center for Neuropsychiatry, China Medical University & Hospital and Beigang Hospital, Taichung and Yun-Lin, Taiwan, ROC

[3]Department of Life Science and Graduate Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan, ROC

[4]Department of Applied Chemistry, Chaoyang University of Technology, Taichung, Taiwan, ROC

[5]Department of Chemistry, National Dong Hwa University, Hualien, Taiwan, ROC

[6]Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan, ROC

#The first two authors contributed equally to this work.

*Correspondence to: Dr. Tzyy-Wen Chiou, PhD

Department of Life Science and Graduate Institute of Biotechnology

National Dong Hwa University, Hualien

No. 1, Sec. 2, Da Hsueh Rd., Shoufeng, Hualien, Taiwan, Republic of China

Tel: 886-3-8633638

Fax: 886-3-8630262

E-mail: twchiou@mail.ndhu.edu.tw

Abstract

We have shown that the natural compound *z*-butylidenephthalide (Bdph), which is 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 polyanhydride material, p(CPP-SA) (Bdph-Wafer) and investigated its antitumor effects. Based on 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 thirtieth day. To investigate the *in situ* antitumor effects of the Bdph-Wafer on glioblastoma multiforme (GBM), we used two 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 against 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.

Running title: *z*-butylidenephthalide wafer to treat human glioblastoma

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

(GBM)

Introduction

Brain tumors occur frequently in humans, with ~2000 new cases of primary central nervous system tumors diagnosed annually in Taiwan alone. Although brain tumors comprise only about 1% of all cancers, they are responsible for 2.5% of the overall cancer 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 plus radiotherapy and eventually chemotherapy the standard treatment [3-6]. Despite this, the median duration of survival of patients with glioblastoma is only 50 weeks. The dismal prognosis of malignant gliomas like 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 their limited therapeutic effects [8-12].

Six major compounds have been isolated from *Angelica sinensis*; (*E*)-liguistilide, (*Z*)-liguistilide, z-butylidenephthalide, palmitic acid, beta-sitosterol, and ferulic acid. *z*-butylidenephthalide (Bdph or K1; molecular weight, ~188.22) and liguistilide (K2; molecular weight, ~190.23) are particularly abundant [13], with the former exhibiting more potency than liguistilide. In conscious rats, Bdph relieves angina without affecting blood pressure or 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 Bdph, significant inhibitory effects on proliferation and cell cycle progression, as well as induction of apoptosis, were found. 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 was increased [17, 18]. Finally, Bdph upregulates 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 anti-cancer 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/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 upregulate genes that promote apoptosis, e.g., 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, which triggers cytochrome c release and apoptosis, which was 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 anti-cancer 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 chemotherapeutical agents to malignant brain tumors. Controlled-release polymers bypass the BBB, preventing systemic toxicity [33]. One such therapy, Gliadel, 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, but only by ~2 months [33]. Side effects do, however, include higher incidences of wound infection and dehiscence [33]. 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 keep it concentration for till 30 days. In speculate, the Bdph-Wafer can increase the local Bdph concentration by 50-fold. Moreover, there was no brain edema, no delay in wound healing, no CSF leakage and no brain infection, a symptom that was observed with the BCNU-Wafer [34, 35]. Furthermore, in a study using a 3% BCNU-Wafer, which is now in clinical use for brain tumor treatment, all mice that underwent treatment (n = 5) died. 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 (Ward Hill, MA, USA). Bdph was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA), incubated with shaking at 25ºC for 1 h, and stored at 4ºC until use. BCNU, sebacic acid (SA), and 1,3-bis (4-carboxyphenoxy) propane (CPP) were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA).

Cell lines and cell culture

DBTRG-05MG human GBM cells and RG2 rat GBM cells were obtained from the American Type Culture Collection (Rockville, MD). G5T/VGH human GBM cells and human GBM 8401 cells were obtained from Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). DBTRG-05MG and human GBM 8401 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, and G5T/VGH and RG2 cells were cultured in DMEM containing 10% fetal bovine serum. Cells were cultured at 37ºC in a humidified atmosphere containing 5% CO_2.

Analysis of cell cytotoxicity

Cell viability was evaluated using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT; Sigma) assay [18]. Briefly, cells were incubated in eight 96-well plates (5×10^3 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 less than or equal to 0.02%. After 24, 48, or 72 h of incubation, the drug-containing medium was replaced with 50 µl 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, Muskegon, MI). The absorbance of untreated cells was set at 100%. The IC_{50} was defined as the concentration that caused a 50% decrease in the absorbance of the drug-treated cells as compared with the untreated cells.

Polymer preparation

SA prepolymer preparation

SA was recrystallized two times in alcohol. SA monomers (2.7 g) were refluxed with 60 ml excess acetic anhydride for 30 min at 135–140ºC 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 150ºC 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 0ºC. 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 180ºC 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 hr, 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 [36]. Bdph was combined with p(CPP:SA) (20:80) to give (by weight) a 3%, 10% and 15% Bdph polymer mixture; a 3% 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 (100 mg final weight) 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 361944 torr. [37-39]. Wafers containing 3% BCNU were prepared by co-dissolving 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 [40]. 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 [40]. Bdph was absorbed at a wavelength of 310 nm, and 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 (10–12 weeks old) 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 (six per group) were given subcutaneous back implants of 1×10^6 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 (five or six per group) also received subcutaneous implants of 1×10^6 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 L × H × W × 0.5236 [41]. 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 (The Ohio State University, Columbus, USA) [42]. 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 three groups as follows: control Wafer group, 10% Bdph-Wafer group and 15% Bdph-Wafer group (n = 6 in each group). Before the wafer implant, mice were anesthetized with an i.p. 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 no. 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 and histological evaluation

At 30 days postimplantation, FGF-SV40 transgenic mice were sacrificed by an i.p. 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 paraffin. The resulting paraffin 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 GFAP. We calculated the tumor volume based on the area of each section and the total coronal section thickness (1 mm) using a microscopy system (Olympus IX70, Japan).

Statistics

Data were expressed as the mean ± S.D. (standard deviation). Statistical significance was analyzed with a Student's *t*-test and Mantel-Cox test. Survival analysis was performed using the Kaplan-Meier method. A value of $P < 0.05$ was considered 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. Intensity of CPP and SA in Fourier transform infrared (FTIR) spectroscopy of CPP-SA copolymer (Fig. 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 6th 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% as 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 show).

Apoptotic pathways and Nur77 translocation induced by Bdph-Wafers

To confirm the results of our previous oligodeoxynucleotide-based microarray analysis [19], we examined the expression of the orphan receptor Nur77 in Bdph-treated RG2 cells using RT-PCR. 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 post-treatment (Fig. 4). 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. 5). Immunoblotting showed that Nur77 was predominantly localized in the nuclear fraction in the absence of Bdph treatment (Fig. 5A). 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. 5A). To further confirm this observation, cytosolic and nuclear fractions were examined using western blot analysis (Fig. 5B). 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. 6A). 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. 6B).

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 1×10^6

RG2 cells and then 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 as compared with either polymer alone or RG2 cells alone (Fig. 7A; $p < 0.005$). The average tumor size at day 30 was 2070.79 ± 784.90 mm^3 in the RG2 group, 1586.30 ± 243.69 mm^3 in the polymer alone group (no drug), 346.71 ± 521.68 mm^3 in the 3% Bdph-Wafer group, 87.89 ± 167.44 mm^3 in the 10% Bdph-Wafer group and 357.48 ± 27.30 mm^3 in the 3% BCNU-Wafer group. In addition, immunohistochemical staining for Ki-67, which indicates cell proliferation [43, 44], showed a substantial decrease in the rats treated with 10% Bdph-Wafers (Fig. 7C). Immunohistochemical staining for caspase-3, which indicates cell apoptosis, showed a substantial increase in the 10% Bdph-Wafer group (Fig. 7C). Finally, no drug-related toxicity was observed in animals given 10% Bdph-Wafers, as evaluated by measurements of body weight (Fig. 7B) and histological analyses of various organs (data not shown). We did, however, observe significant weight loss in animals treated with 3% BCNU-Wafers (Fig. 7B).

Therapeutic effects of Bdph-Wafers on xenograft tumor growth

To determine whether Bdph-Wafers suppress human GBM tumor growth, nude mice were inoculated with 1×10^6 human DBTRG-05MG cells and implanted with 3% and 10% Bdph-Wafers or with control wafers on day 5. 3% and 10% Bdph-Wafers inhibited tumor growth (Fig. 8). Significant suppression of tumor growth in the 3% and 10% Bdph-Wafer groups was observed (Fig. 8A). The mean values for tumor size at day 39 were 1098.46 ± 170.11 mm^3 in the control group, 627.71 ± 137.44 mm^3 in the 3% Bdph-Wafer group, and 504.4 ± 150.79 mm^3 in the 10% Bdph-Wafer group (Fig. 8A; $p < 0.01$ control vs. 3% and 10% Bdph-Wafers).

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 sacrificed 20 days after wafer implantation, and coronal sections from each mouse brain were stained with H&E. The average tumor volume 20 days after the wafer was implanted was 0.33 ± 0.33 mm^3 for the control group as compared with 0.16 ± 0.22 mm^3 for the 10% Bdph-Wafer group and 0.12 ± 0.02 mm^3 for the 15% Bdph-Wafer group. Furthermore, the relative tumor volume was reduced by 51% in the 10% Bdph-Wafer group and 64% in the 15% Bdph-Wafer group (Fig. 9A). There was a significant inhibitory effect on tumor growth for both the 10% and 15% Bdph-Wafer groups as compared with the control (wafer-only) group ($P < 0.05$; Fig. 9 B). 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. 9C). 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. 9C). Finally, tumor volume was reduced by the Bdph-Wafers in a dose-dependent manner (Fig. 9A).

Discussion

Telomerase is widely expressed in 80–90% of human cancers, but it is almost undetectable in normal somatic cells [45]. 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 [46]. 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) [45]. This suggests that telomerase activation is involved in the establishment of cellular immortality and may therefore be a critical step in carcinogenesis [47]. Therefore, inhibiting telomerase activity may be a therapeutic

strategy for selectively targeting malignant gliomas and sparing normal brain tissue [48].

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, resulting 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 side effects if Bdph is systemically administered to treat malignant gliomas; this may pose a particular problem for long-term treatments. To attempt to solve this potential issue, we tested the local, controlled release of drugs bound to polymers. Speculation, our Bdph-Wafer increased the local Bdph concentration 50-fold but did not lead to inhibition of telomerase activity in germ cells or bone marrow cells. 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.

The carmustine implant, a currently used local delivery Gliadel wafer, is an alkylating agent that kills both tumor and normal cells. As a consequence, many side 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 LD50. The reason for its mild side effects is because Bdph is 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]. Nur77 has been implicated in growth inhibition and apoptosis [19, 21, 23, 26-28, 32], suggesting that Nur77 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 that Bdph has on tumor viability (Fig. 3), signal transduction (Fig. 6), Nurr77 protein translocation (Fig. 5), and histochemical staining of in vivo brain tumor cell xenografts (Figs. 7) when it is released from biodegradable wafers. Our results implicated the PKC pathway in signal transduction (Fig. 6A), showed Nur77 translocation from the nucleus to the cytoplasm (Fig. 5), and demonstrated caspase expression leading to tumor cell apoptosis (Fig. 7C). These results are consistent with the effects of Bdph when not incorporated into polymer form. Our findings suggest that the Bdph-Wafer retains its natural biological antitumor function.

Kadota et al. (2000) have reported the biodistribution of Bdph. Less than 1 h after dermal application, labeled Bdph (unchanged [8-^{14}C]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 was decreased. The biodistribution data showed that only 0.029 μg/g of brain tissue was detected after 1 h [51]. 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 about 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.

Kadota et al. (2000) have 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 [51]. 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 (i.e., 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 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 PLGA, 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. 9). 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 [34, 35]. This may be because Bdph is less toxic (LD50, 7.5 g/kg) as compared with 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. As 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 against 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.

Previously, we showed that 300 mg/kg Bdph, when administered s.c., reduced tumor volume by ~25% at day 15 [18], whereas 15% Bdph-Wafers (15 mg Bdph) implanted into the brains of FGF-SV40 transgenic mice decreased tumor size by ~64% at day 20. We therefore estimate that our Bdph-Wafer interstitial controlled-release device can deliver an anti-tumor Bdph effect that is 50 times more potent than s.c. injection.

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 [19]. According to a pharmacokinetic study, the half life of Bdph is around 1 day, with excretion from urine [51]. In this wafer-based system, Bdph is slowly released for up to 30 days (Fig. 2). More importantly, at 30 days post-implantation, Nurr77 translocation and tumor cell apoptosis were still observed in our nude mouse model (Fig. 7C). 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 gene-targeting drug, increase the local Bdph concentration and maintain its stability, leading to significant inhibition of tumor growth.

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Figure legends

Fig. 1. Structural analysis of the CPP-SA copolymer. (A) ^{1}H NMR spectroscopy of the CPP-SA copolymer. The characteristic signals of aromatic protons in CPP were observed at 6.9–8.2 ppm. Another characteristic signal of methylene protons in SA was measured at 1.3 ppm. (B) FTIR spectroscopy of the CPP-SA copolymer. The characteristic signal of the anhydride bond between CPP and SA (shown circled in red) was observed at 1812.76 cm^{-1}.

Fig. 2. The release kinetics of 10% and 15% Bdph-Wafers were measured over 30 days in vitro. Sustained release of Bdph was observed with both wafer formulations. *$p < 0.05$. n=3 for each concentration.

Fig. 3. Growth inhibition of rat malignant glioma cells by Bdph-Wafers. (A) The rat malignant glioma cell line RG2 was treated with control wafers and with 3% and 10% Bdph-Wafers for 24 h. Cell viability was determined with the MTT assay. Data shown are the mean ± S.D. from three independent experiments. **$p <$ 0.01. (B) Cell morphology of RG2 malignant glioma cells treated for 24 h with control wafers and with 3% and 10% Bdph-Wafers.

Fig. 4. Induction of Nur77 transcripts after Bdph exposure. Rat GBM cells (RG2) were treated with Bdph (100 μg/ml) for the indicated time periods (0, 0.5, 1, 3, and 6 h). CD437 (1 μM) was used as the Nur77 positive control. After incubation with drugs, cells were collected, and total RNA was isolated. GAPDH was used as an internal control.

Fig. 5. Bdph induced Nur77 to migrate from the nucleus to the cytoplasm. (A) Rat GBM cells (RG2) cells were treated with Bdph (100 μg/ml) for 24 h and then immunostained with Nur77 antibody followed by the corresponding rhodamine-conjugated anti-IgG secondary antibody to show Nur77 protein localization. Nuclei were stained with DAPI. Staining was visualized with a fluorescence microscope (bar = 50 μm). (B) Bdph induced Nur77 translocation from the nucleus to the cytoplasm in RG2 cells. RG2 cells were plated in 10-cm dishes and incubated until 90% confluent. Cells were treated with Bdph (100 μg/ml) for different time periods (0, 6, 12, 24, and 48 h). The cells were harvested and nuclear and cytoplasmic fractions were isolated. CD437 (1 μM) was used as the Nur77 positive control. Nur77 expression in cytoplasmic and nuclear fractions was evaluated by western blot. β-actin was used as an internal control.

Fig. 6. Role of signaling pathways in Bdph-induced growth inhibition. (A) RG2 cells were treated with Bdph (100 μg/ml) for 0 to 180 min as indicated. Western blot analysis was performed with pPKC, pJNK, JNK, pERK, ERK, pAKT and AKT antibodies. Expression of β-actin was used as an internal control. (B) RG2 cells were pretreated with a 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) for 1 h. Subsequently, the RG2 cells were treated with 100 μg/ml Bdph for 24 h. The viability was determined with an MTT assay. *$p < 0.05$.

Fig. 7. Bdph-Wafers inhibited tumor growth in a syngeneic rat GBM model. RG2 cells (1×10^6) 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 ± S.D. *$p < 0.05$. (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 (400×; bar = 50 μm).

Fig. 8. Bdph-Wafers inhibited tumor growth in a human GBM xenograft nude mouse model. DBTRG-05MG cells (1×10^6) were implanted subcutaneously into the hind flank region of Foxn1 nu/nu mice. Five days after transplantation, mice were treated with wafers only or with 3% and 10% Bdph-Wafers. Tumor sizes (A) and body weights (B) are shown as the mean ± S.D. *$p < 0.05$. **$p < 0.01$ (C) Tumors isolated from animals treated with wafers only or with 3% and 10% Bdph-Wafers.

Fig. 9. Bdph-Wafers inhibited tumor growth in a spontaneous brain tumor mouse model.

FGF-SV40 transgenic mice were implanted with control, 10% or 15% Bdph-Wafers to verify antitumor activity of Bdph. Twenty days after wafer implantation, the mice were sacrificed, and the tumors were analyzed. (A) The relative tumor area ± S.D. is shown for each group (*$p < 0.05$). (B) Hematoxylin and eosin (H&E) staining of coronal sections (200X; bar = 100 μm). (C) Immunohistochemical staining for GFAP. Positive cells are stained brown (100X; bar = 100 μm). Arrow head indicates the area of tumor.

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Department of Life Science and Graduate Institute of Biotechnology National Dong Hwa University, Hualien No. 1, Sec. 2, Da Hsueh Rd., Shoufeng, Hualien, Taiwan, Republic of China

Address

886-3-8633638 | twchiou@mail.ndhu.edu.tw

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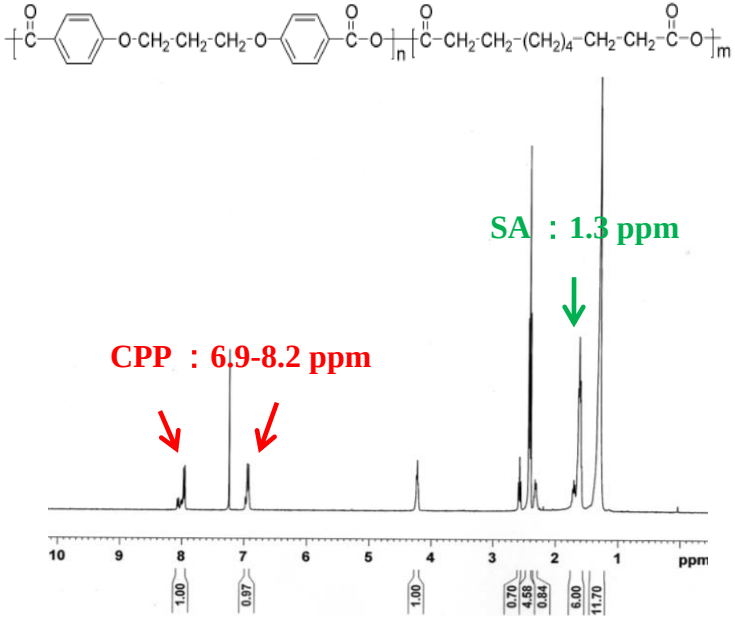
Please sign and return original form to the Editorial Office; a photocopy should be retained for your files.

Figure

Fig. 1

A



B

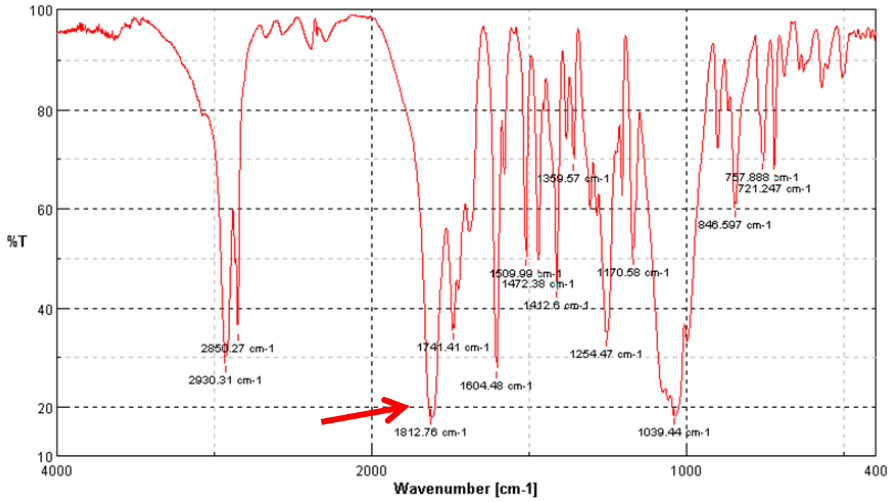


Fig. 2

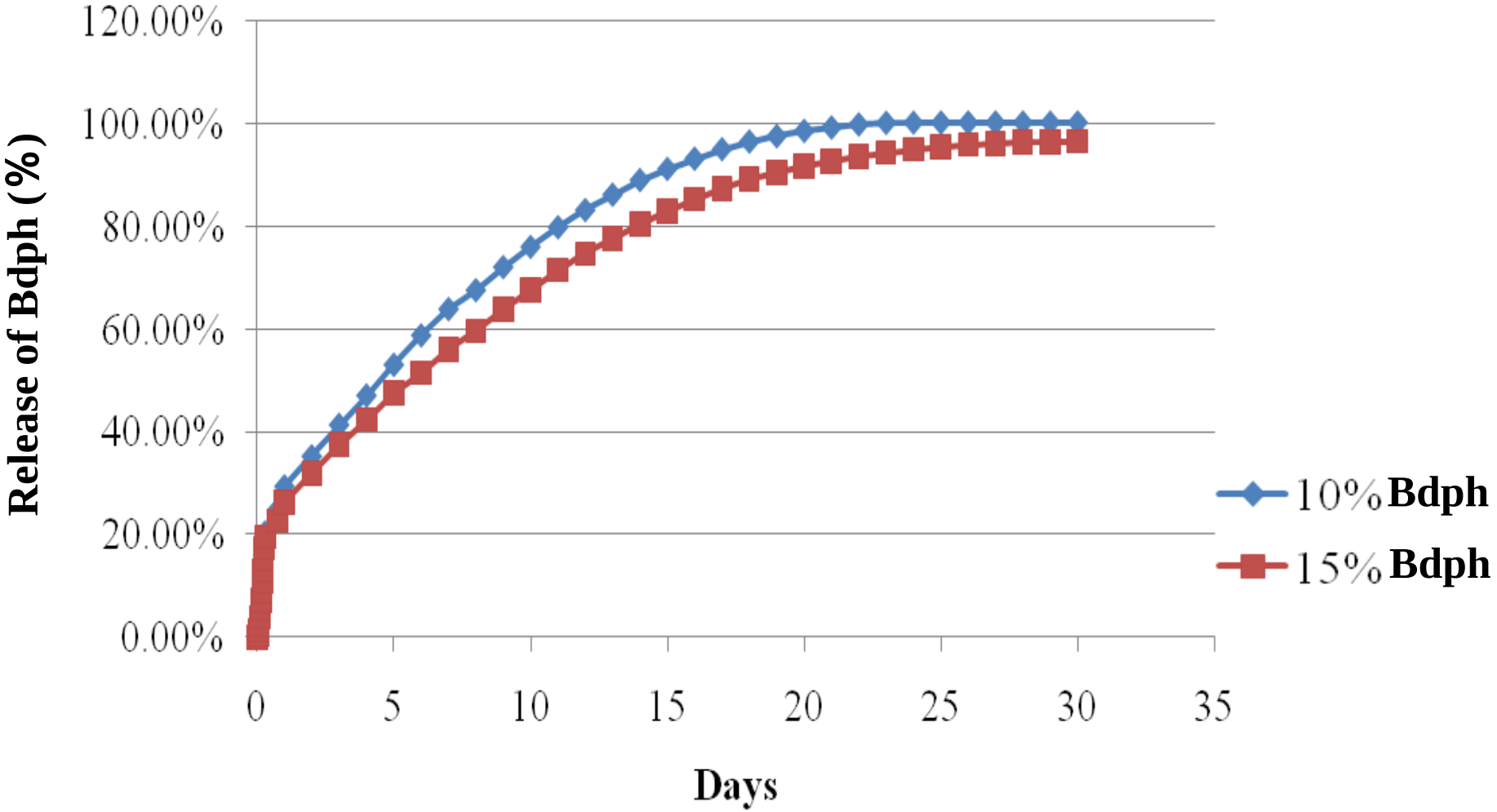
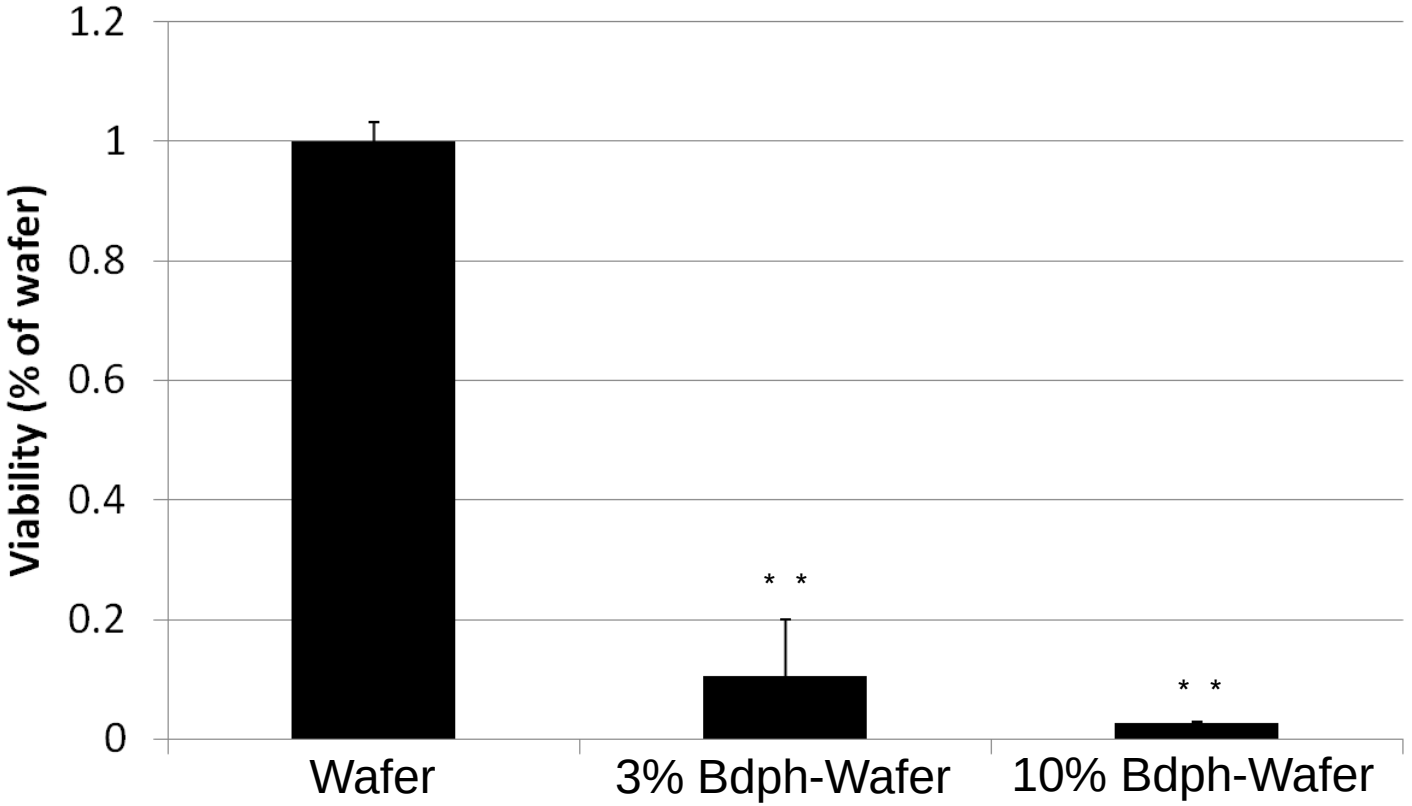
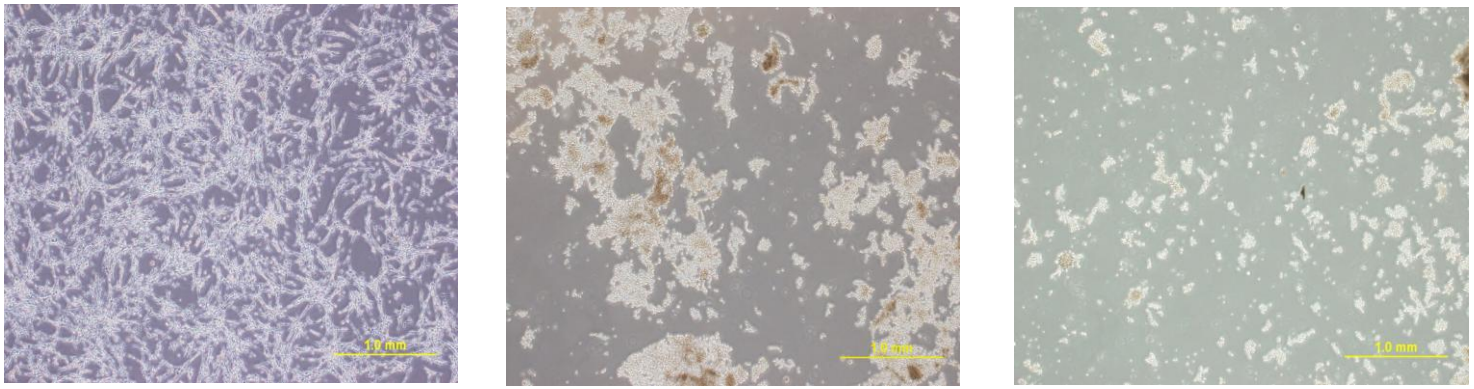

120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Release of Bdph (%)
0
5
10
15
20
25
30
35
Days
10% Bdph
15% Bdph

Fig. 3

A



B



Wafer | 3% Bdph-Wafer | 10% Bdph-Wafer

Fig. 4

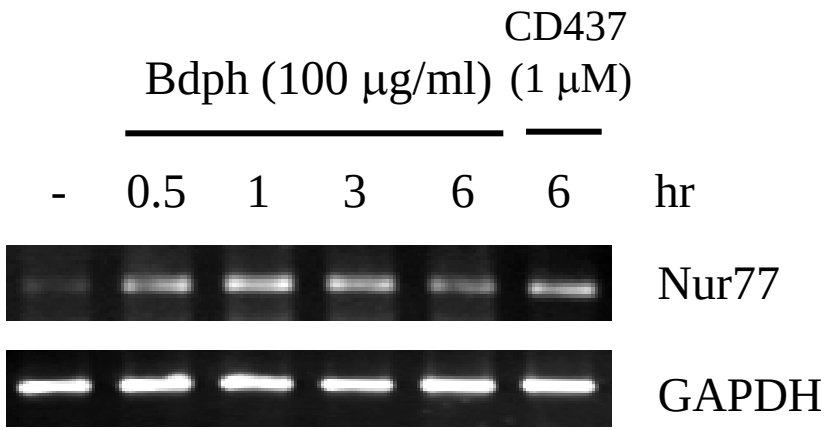
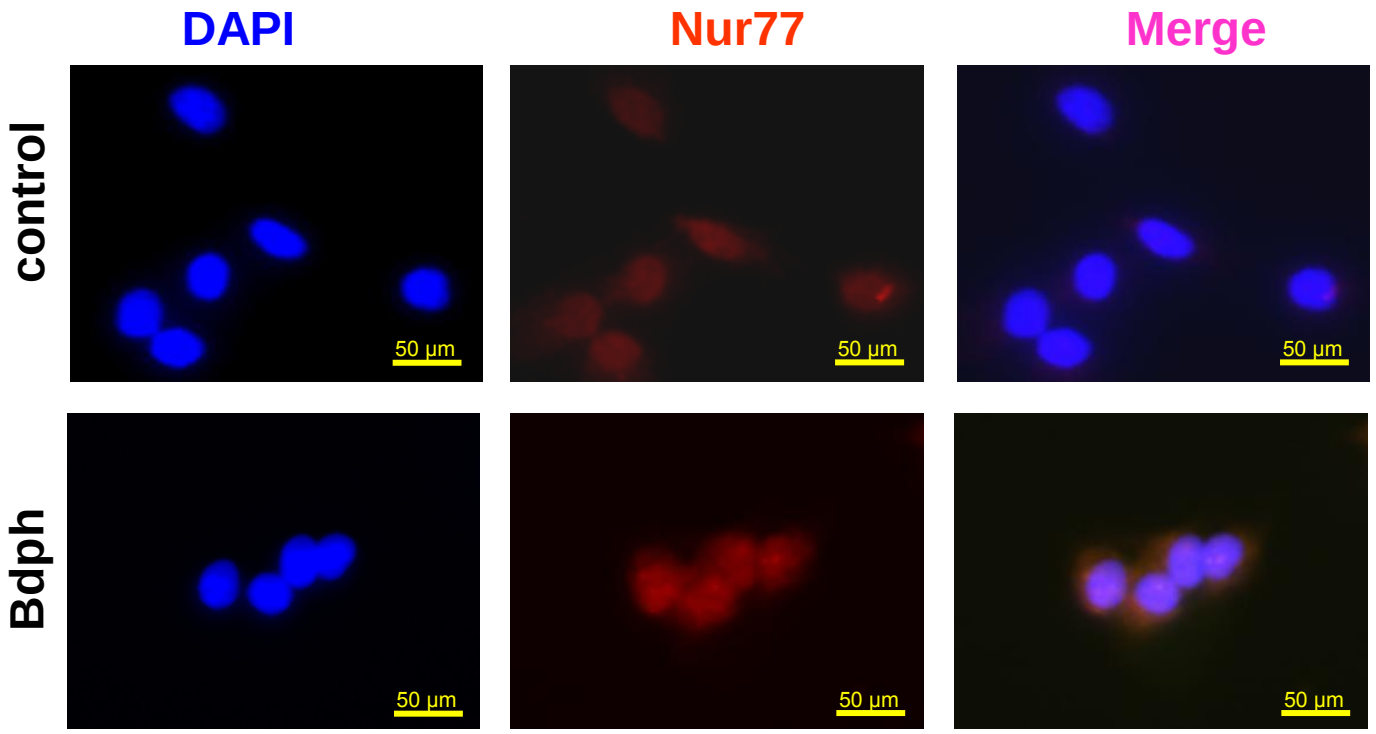


Fig. 5 A


DAPI
Nur77
Merge
control
Bdph
50 μm
50 μm
50 μm
50 μm
50 μm
50 μm

B

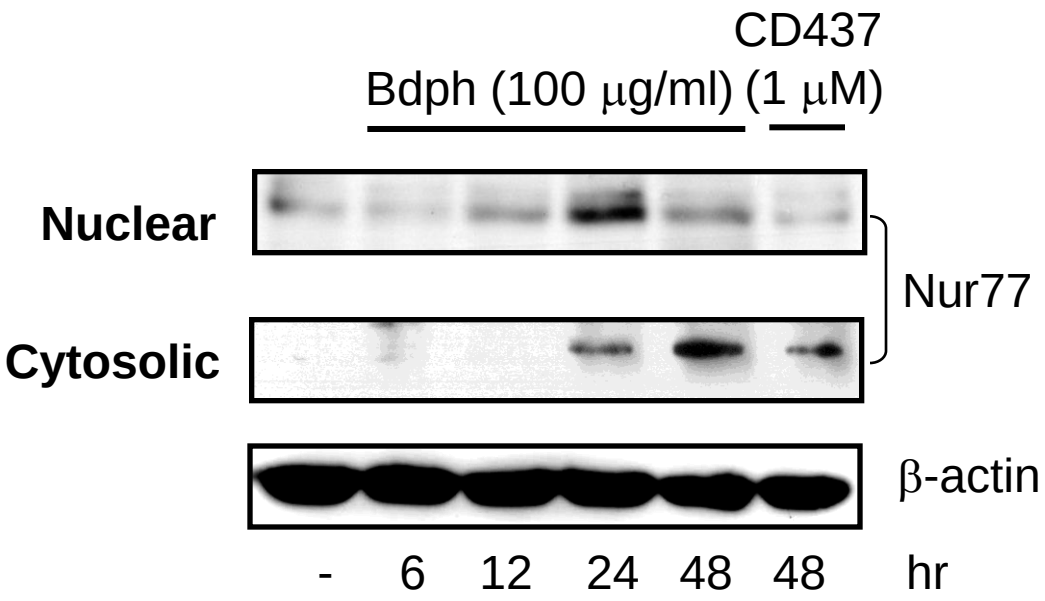

CD437
Bdph (100 μg/ml) (1 μM)
Nuclear
Cytosolic
Nur77
β-actin
- 6 12 24 48 48 hr

Fig. 6

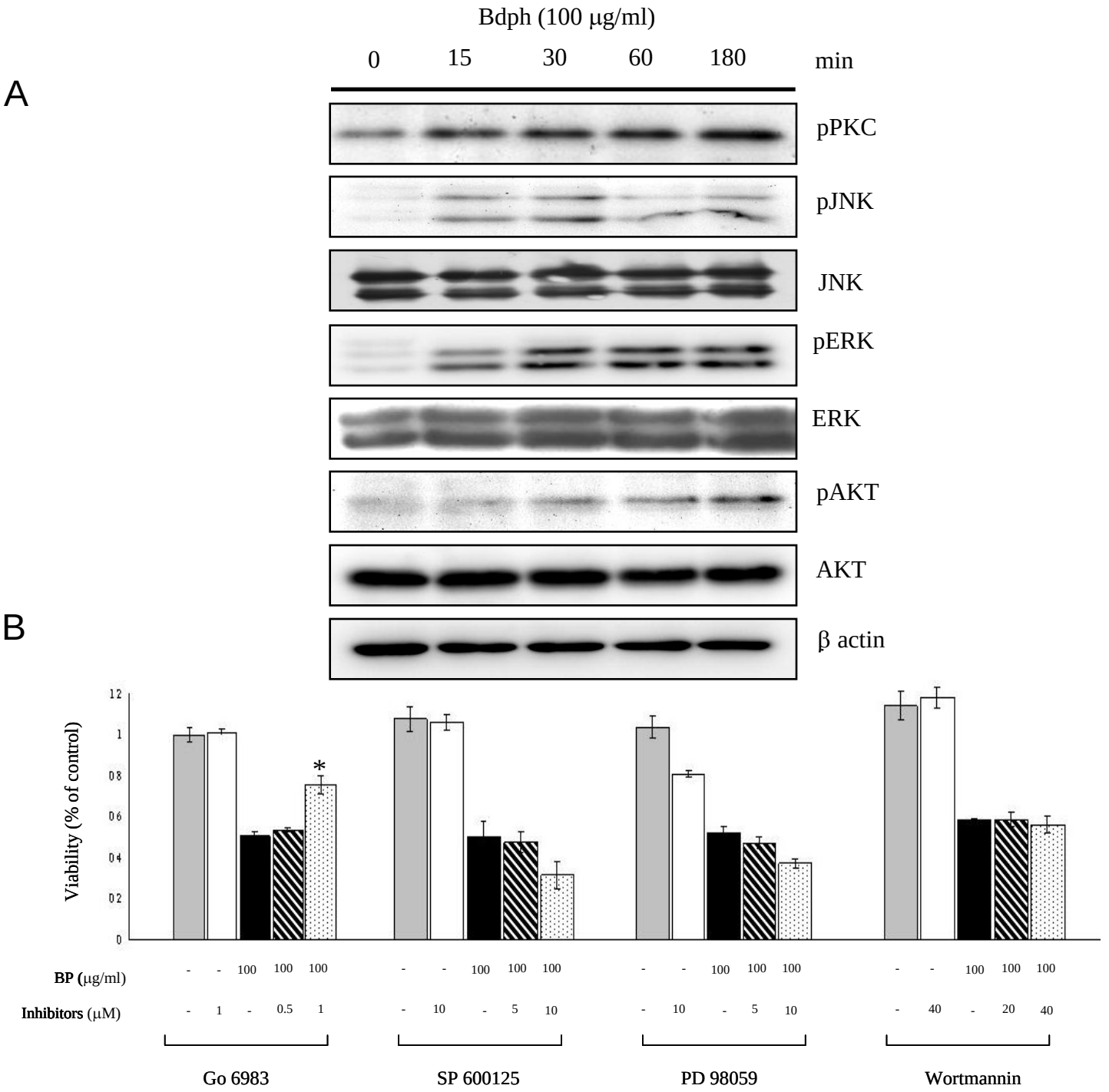

A
Bdph (100 μg/ml)
0
15
30
60
180
min
pPKC
pJNK
JNK
pERK
ERK
pAKT
AKT
β actin
B
Viability (% of control)
12
1
0.8
0.6
0.4
0.2
0
*
BP (μg/ml)
- - 100 100 100 - - 100 100 100 - - 100 100 100 - - 100 100 100
Inhibitors (μM)
- 1 - 0.5 1 - 10 - 5 10 - 10 - 5 10 - 40 - 20 40
Go 6983
SP 600125
PD 98059
Wortmannin

Fig. 7

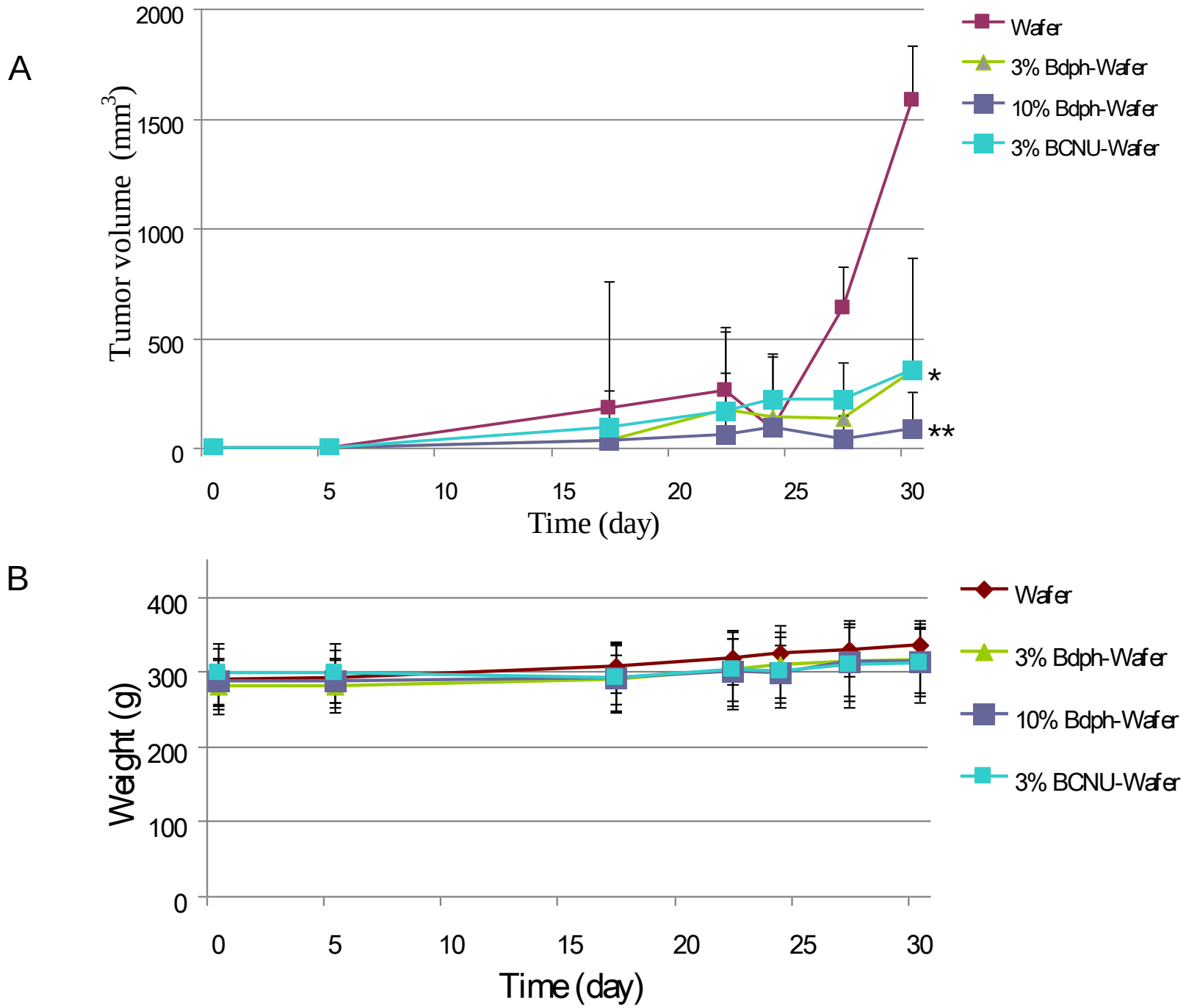

A
2000
1500
1000
500
0
Tumor volume (mm3)
0
5
10
15
20
25
30
Time (day)
Wafer
3% Bdph-Wafer
10% Bdph-Wafer
3% BCNU-Wafer
*
**
B
400
300
200
100
0
Weight (g)
0
5
10
15
20
25
30
Time (day)
Wafer
3% Bdph-Wafer
10% Bdph-Wafer
3% BCNU-Wafer

Fig. 7

C

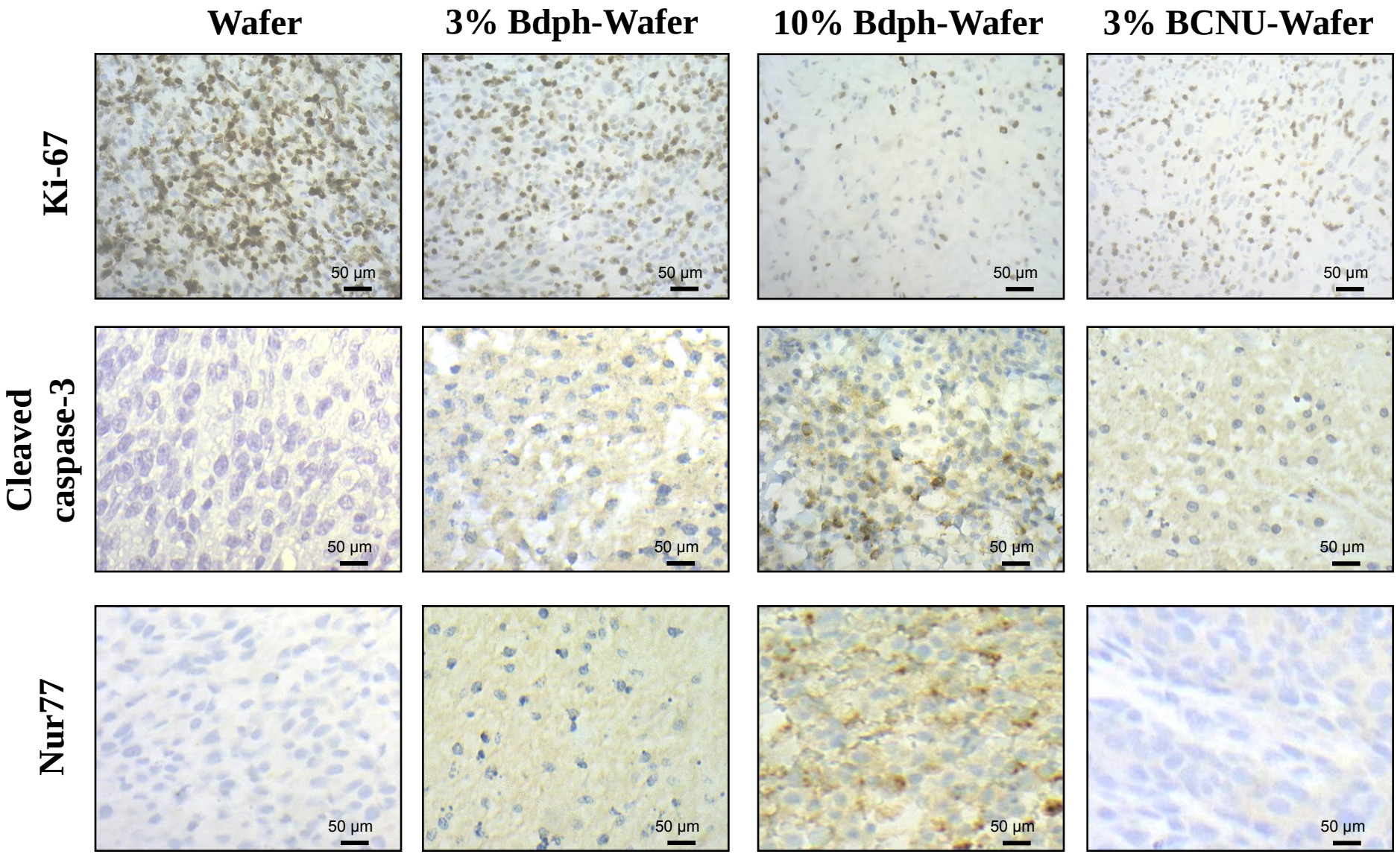

Wafer
3% Bdph-Wafer
10% Bdph-Wafer
3% BCNU-Wafer
Ki-67
Cleaved caspase-3
Nur77
50 μm

Fig. 8

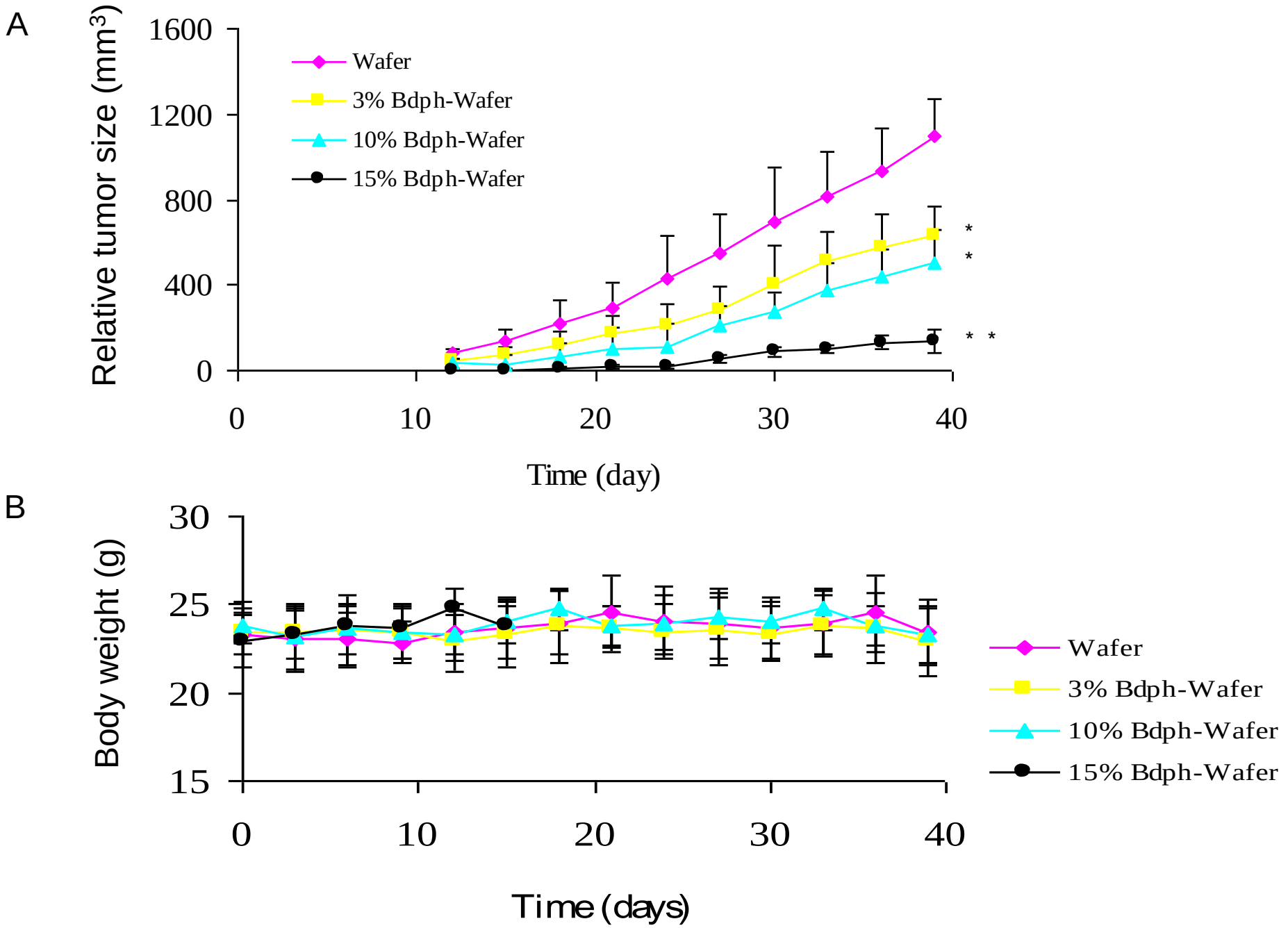
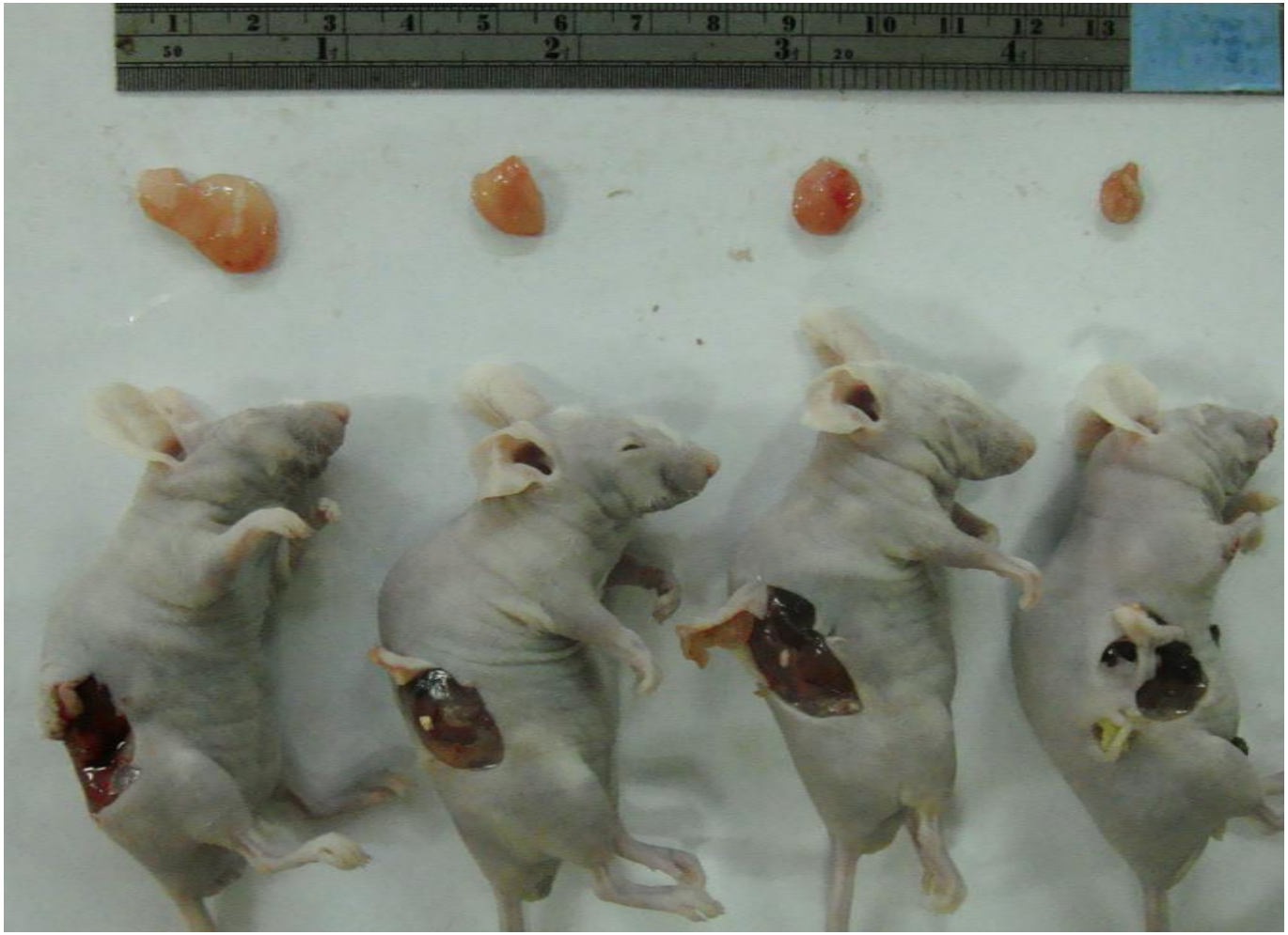

A
Relative tumor size (mm3)
1600
1200
800
400
0
Wafer
3% Bdph-Wafer
10% Bdph-Wafer
15% Bdph-Wafer
* *
* *
0
10
20
30
40
Time (day)
B
Body weight (g)
30
25
20
15
0
10
20
30
40
Time (days)
Wafer
3% Bdph-Wafer
10% Bdph-Wafer
15% Bdph-Wafer

Fig. 8

C



Wafer

3%
Bdph-Wafer

10%
Bdph-Wafer

15%
Bdph-Wafer

Fig. 9

A

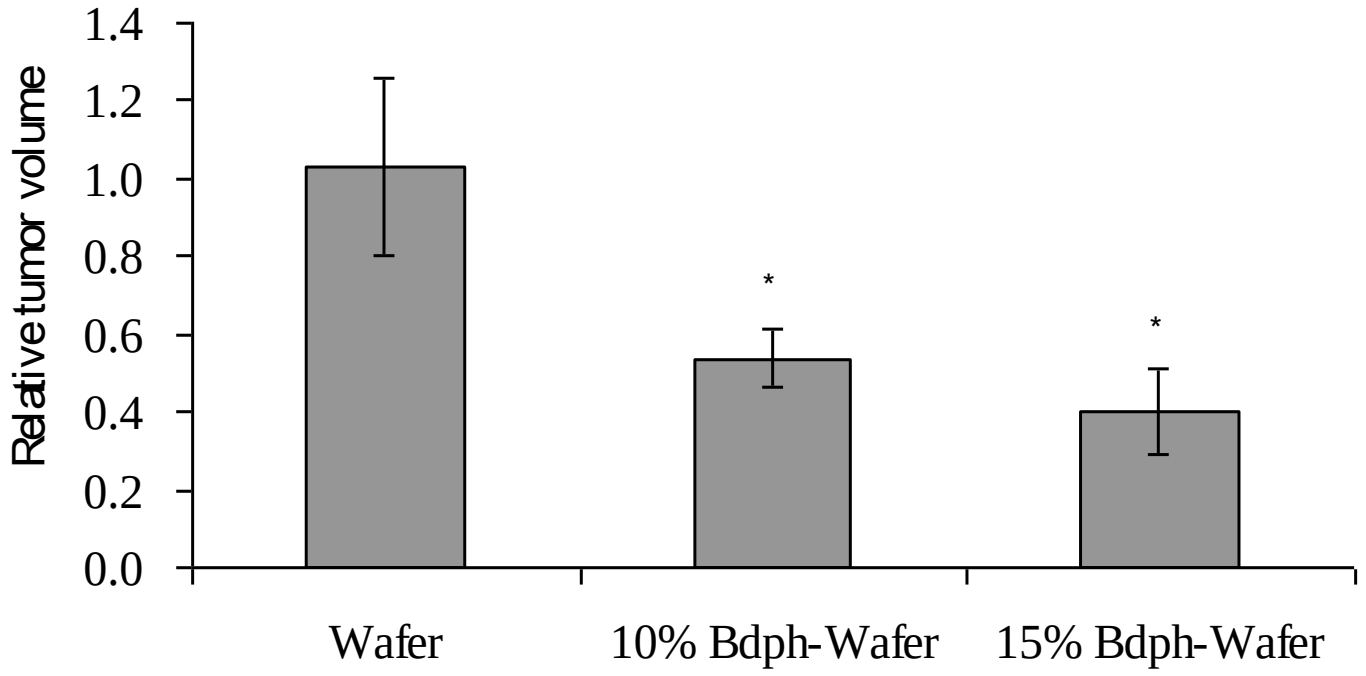
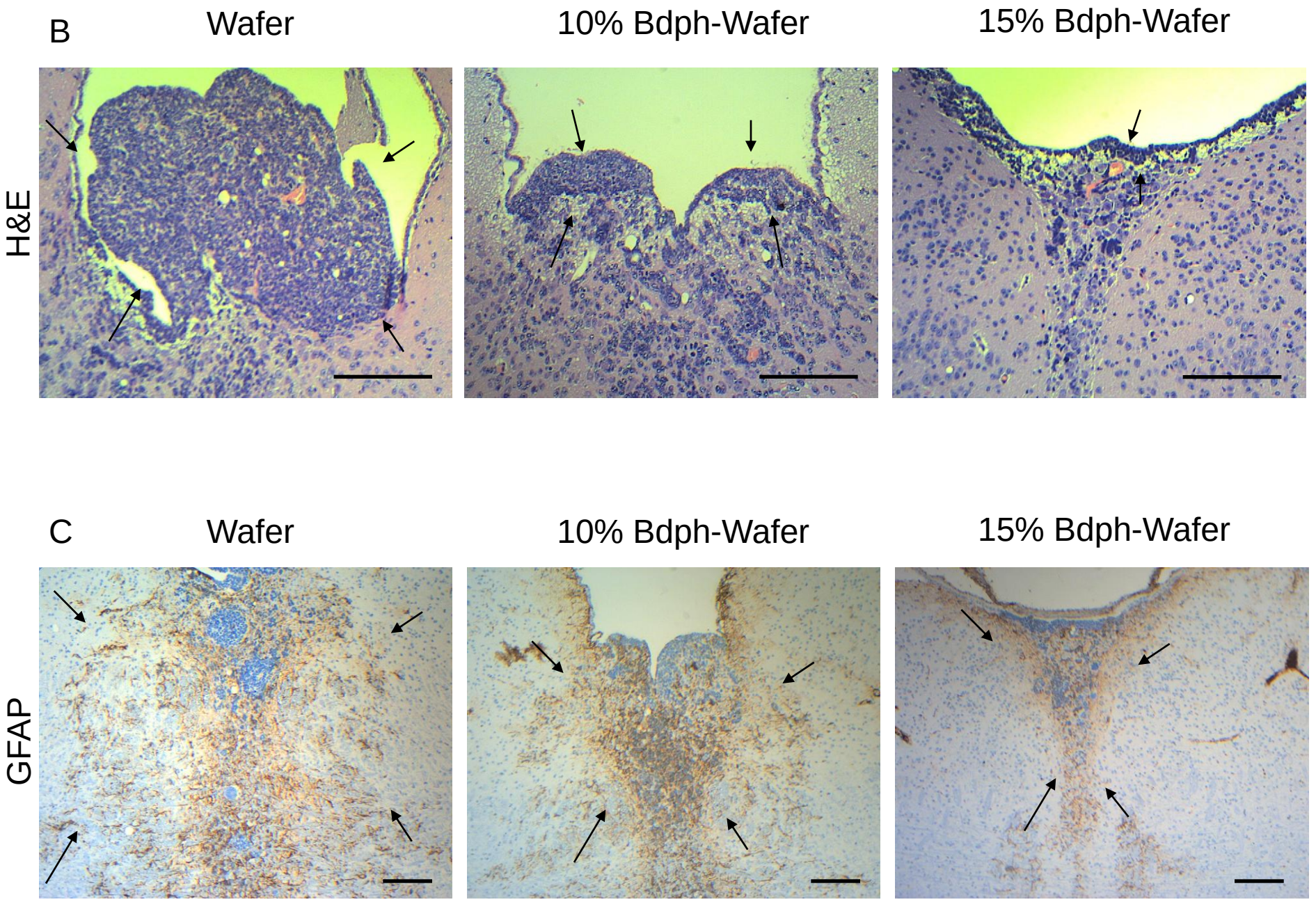

Relative tumor volume
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Wafer
10% Bdph-Wafer
15% Bdph-Wafer

Fig. 9


B
Wafer
10% Bdph-Wafer
15% Bdph-Wafer
H&E
C
Wafer
10% Bdph-Wafer
15% Bdph-Wafer
GFAP