

ORIGINAL ARTICLE

Biodegradable interstitial release polymer loading a novel small molecule targeting Axl receptor tyrosine kinase and reducing brain tumour migration and invasion

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Glioblastoma multiforme (GBM) is the most common and aggressive brain tumour. The neoplasms are difficult to resect entirely because of their highly infiltration property and leading to the tumour edge is unclear. Gliadel wafer has been used as an intracerebral drug delivery system to eliminate the residual tumour. However, because of its local low concentration and short diffusion distance, patient survival improves non-significantly. Axl is an essential regulator in cancer metastasis and patient survival. In this study, we developed a controlled-release polyanhydride polymer loading a novel small molecule, *n*-butylideneophthalide (BP), which is not only increasing local drug concentration and extending its diffusion distance but also reducing tumour invasion, mediated by reducing Axl expression. First, we determined that BP inhibited the expression of Axl in a dose- and time-dependent manner and reduced the migratory and invasive capabilities of GBM cells. In addition, BP downregulated matrix metalloproteinase activity, which is involved in cancer cell invasion. Furthermore, we demonstrated that BP regulated Axl via the extracellular signal-regulated kinases pathway. Epithelial-to-mesenchymal transition (EMT) is related to epithelial cells in the invasive migratory mesenchymal cells that underlie cancer progression; we demonstrated that BP reduced the expression of EMT-related genes. Furthermore, we used the overexpression of Axl in GBM cells to prove that Axl is a crucial target in the inhibition of GBM EMT, migration and invasion. In an *in vivo* study, we demonstrated that BP inhibited tumour growth and suppressed Axl expression in a dose-dependent manner according to a subcutaneous tumour model. Most importantly, in an intracranial tumour model with BP wafer *in situ* treatment, we demonstrated that the BP wafer not only significantly increased the survival rate but also decreased Axl expression, and inhibited tumour invasion. These results contribute to the development of a BP wafer for a novel therapeutic strategy for treating GBM invasion and increasing survival in clinical subjects.

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INTRODUCTION

Glioblastoma multiforme (GBM) and malignant gliomas are the most common and aggressive brain tumours because of their highly vascularised and invasive neoplasms.^{1,2} The diffusely invasive properties of malignant gliomas make them nearly impossible to totally resect; thus, surgery plus radiotherapy and eventual chemotherapy are the standard treatment.^{3–6} The therapies available for treating these neoplasms are limited, and the prognosis is extremely poor. In recent years, new strategies have included developing drugs considered to be effective therapeutic approaches to GBM growth, invasiveness and vascularisation. In clinical treatment, interstitial chemotherapy with biodegradable Gliadel wafer, which is loading with carmustine (BCNU), has been performed. Robert S Langer is the developer of Gliadel wafer and has pointed out that the limitation of Gliadel wafer is the short penetration distance (< 2 mm) of BCNU from the wafer; it may be the reason why it is difficult to completely eradicate a residual tumour, and tumour invasion is poorly

inhibited. Consequently, we are developing a novel small molecule that can selectively target oncogenic pathways and diffuse over long distances that constitute a new strategy for GBM treatment. Kinases are the most common class of proteins targeted by these new drugs.

Axl receptor tyrosine kinase (RTK) has been shown to be linked with various high-grade cancers and related to poor diagnosis;⁷ in addition, it has been suggested to be involved in several cellular responses, including cell survival, cellular adhesion, proliferation, autophagy, migration, invasion, angiogenesis, platelet aggregation and natural killer cell differentiation.^{8,9} Axl was originally identified as a transforming gene in chronic myeloid leukaemia.^{8,10} Moreover, Axl activation has been associated with several signal transduction pathways, including Akt, mitogen-activated protein kinase, nuclear factor- κ B and signal transducer and activator of transcription.^{11,12} Recently, several RTKs have been actively pursued as targets for therapeutic intervention.

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The epithelial-to-mesenchymal transition (EMT) has been demonstrated in organ fibrosis and in the initiation of metastasis for cancer progression.¹³ The EMT programme is activated by Twist and the Snail family transcription factors Snail (Snail/SNAI1) and Slug (Slug/SNAI2). These EMT transcription factors adjust the epithelial gene expression, determined by the suppression of genes encoding epithelial junctional complexes (for example, E-cadherin and β -catenin) and cytokeratin, and induce the expression of mesenchymal vimentin and N-cadherin.^{14,15} In 2008, a study demonstrated that Axl RTK is frequently over-expressed in both glioma and vascular cells and predicts poor prognosis in GBM patients.¹⁶ In addition, several scientists have determined that Axl RTK has the essential role of an EMT-induced regulator in tumour formation, cancer metastasis and patient survival.^{17,18} Furthermore, in our previous microarray data, we determined that Axl RTK was considerably downregulated after BP treatment.¹⁹

In this study, we developed a controlled-release 1, 3-bis-(p-carboxyphenoxy)propane-co-sebacic acid (CPP-SA) polymer loading a novel small molecule, *n*-butylideneephthalide (BP), not only increasing local concentration and extending its diffusion distance but also reducing tumour cell invasion mediated by reducing Axl expression. In our previous study, we proved that BP has antitumour effects on GBM brain tumours *in vitro* and *in vivo*.^{20,21} In 2008, we discovered a possible chemotherapy target in GBM.¹⁹ In addition, our team observed that BP inhibited telomerase activity and promoted GBM cell senescence.^{22,23} High expression of MGMT (O-6-methylguanine-DNA methyltransferase) in cancer cells may lead to blunting the therapeutic effect of temozolomide (TMZ). Recently, we demonstrated that BP has a combination effect with TMZ and reduces MGMT expression to overcome TMZ drug resistance.²⁴ In addition, we developed a local drug delivery polymer-BP wafer;²⁵ we demonstrated that the BP wafer released 90% of the available BP by the 30th day and proved that the released BP from a 10% BP wafer inhibited the growth of malignant glioma RG2 cells by 90%, compared with control wafer (0% BP wafer). Our BP wafer significantly reduced the tumour size in a dose-dependent manner in a subcutaneous (SC) tumour model (F344 rat and nude mice) and in FGF-SV40 spontaneous brain tumour transgenic mice. These findings indicate that BP is a promising new anticancer compound with the potential for clinical application.

In this study, we aimed to determine the target that is involved in GBM migration and invasion. We demonstrated that the small-molecule drug BP reduced Axl expression and observed that Axl played a role in mediating glioma cell proliferation, cell migration and invasion. In an *in vivo* experiment, we developed a local delivery system: a BP wafer was administered in a high dose. The system prolonged rat survival 2.44-fold compared with a Gliadel wafer. BP targeting of Axl may represent a promising new method for intervening in GBM progression and invasion.

RESULTS

BP regulates axl expression in human GBM cells

In our previous study, we demonstrated the antitumour effects of BP on GBM cells both *in vitro* and *in vivo*. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that BP exerted an antiproliferative effect on GBM cells; the IC₅₀ concentration of DBTRG-05MG was 100 ± 3 µg/ml. In addition, we used oligodeoxynucleotide-based microarray screening to identify BP-mediated changes in gene expression.

To confirm the microarray results, we examined the expression of Axl in BP-treated DBTRG-05MG through reverse transcriptase-PCR and western blotting. In cells treated with BP at its 100 µg/ml, the mRNA expression of Axl was suppressed in a time-dependent manner (Figure 1a). In addition, the protein expression of Axl was

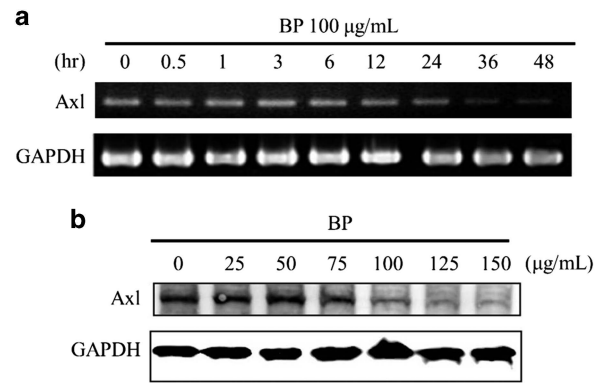


Figure 1. BP regulates Axl expression in human GBM cells. The reverse transcriptase-PCR and western blot data demonstrate that BP can downregulate Axl protein expression in DBTRG brain tumour cells in a time- or dose-dependent manner. (a) Time-dependent downregulation in treatment with BP at 100 µg/ml and (b) dose-dependent downregulation in 24-h treatment with BP. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

downregulated in DBTRG-05MG in a dose-dependent manner after BP treatment (Figure 1b). Axl was significantly decreased after BP treatment. Axl is involved in cell migration and invasion; therefore, we evaluated the possibility of inhibiting cell migration and invasion through BP treatment.

BP inhibits human GBM cell migration and invasion

As shown in Figure 1, BP downregulated Axl expression in human GBM cells. Axl is known as a cancer cell migration and invasion correlation gene; therefore, we used various methods to evaluate the effect of BP on the invasion and migration of DBTRG-05MG in human GBM cells. First, we used a wound-healing assay, which is a simple, inexpensive and originally developed method, to determine whether BP inhibited cell migration in the GBM cell line DBTRG-05MG. The cells were grown in a culture dish until reaching a confluent phase; subsequently, 'wounds' were induced by scraping a line with a plastic tip. The healing of the wound margins was analysed within 24 h after drug treatment. Photomicrographs depicted in Figure 2a show that the number of migrating cells decreased after BP treatment in a dose-dependent manner. The 50 µg/ml group exhibited significantly delayed wound closure. In addition, we used Transwell filters with or without Matrigel coating to assess the cell migration and invasion ability after BP exposure. The results showed that BP can not only stop cell migration (Figure 2b) but also inhibit cell invasion (Figure 2c).

Because matrix metalloproteinases (MMPs) have a crucial role in tumour invasion and metastasis processes, we used a zymography assay to detect extracellular matrix (ECM) hydrolytic enzymes. We determined that BP significantly influenced the expression levels of MMP (Figures 3 and 4). This revealed that BP mediates cell migration and invasion by inhibiting ECM hydrolytic enzyme activity.

Axl is required for mediating the inhibition of human GBM cell proliferation, migration, invasion and EMT by BP

To examine the functional importance of Axl expression mediated by BP in tumour cell growth, migration and invasion, we induced Axl overexpression in cells by transfecting them with the plasmid pcDNA3.0-Axl. After colony selection, we obtained pcDNA3.0-neo and pcDNA3.0-Axl DBTRG cell lines (Figure 5a). Figure 5b shows that the viability of cells with Axl overexpression recovered completely after BP exposure compared with the viability of

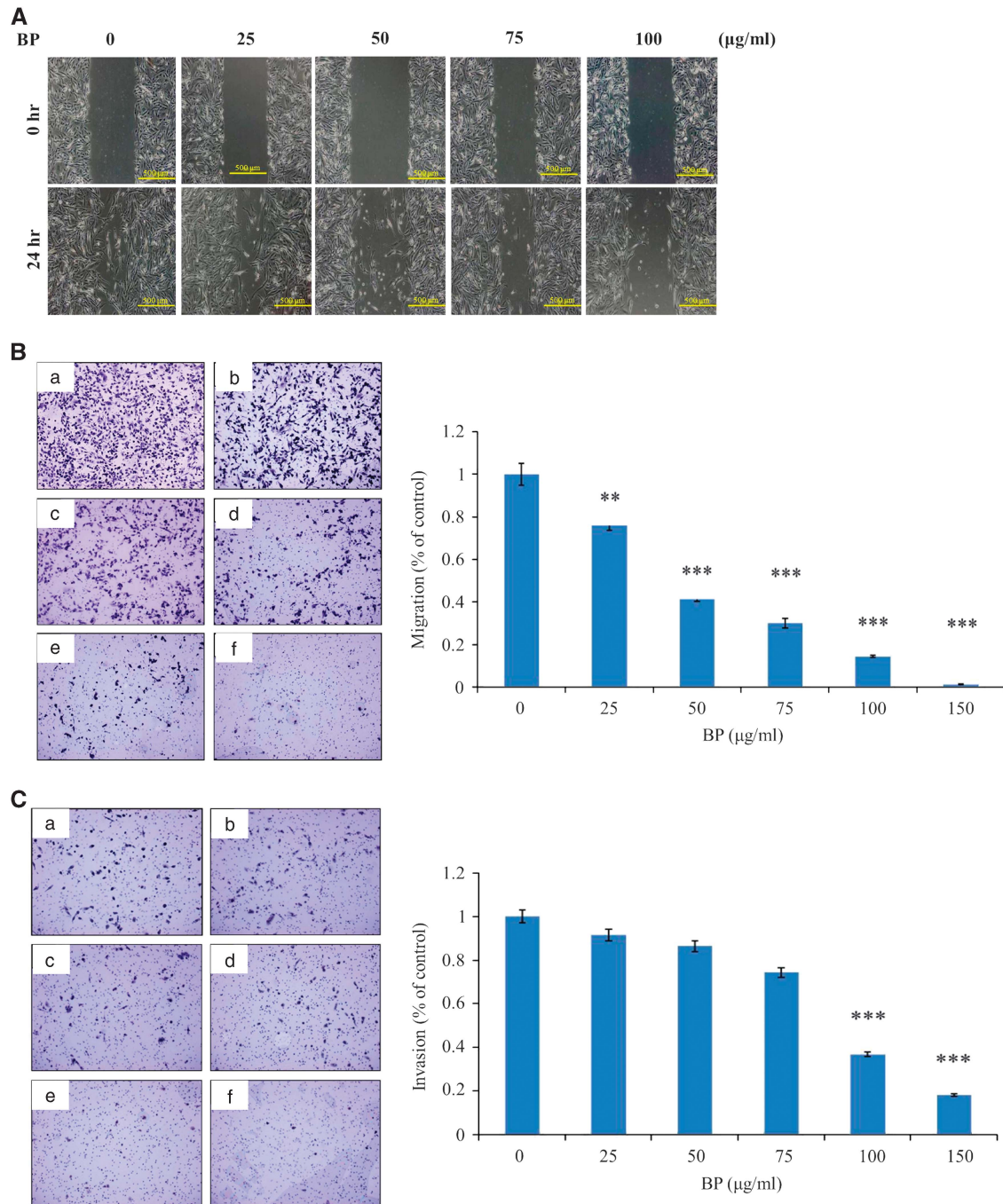


Figure 2. BP inhibits migration and invasion in the GBM cell line DBTRG-05MG in a dose-dependent manner. (A) Wound-healing assay of DBTRG-05MG cells treated with BP at various doses in 24-h treatment. Transwell assay for migration (B) and invasion (C) with BP at various doses in 24-h treatment: (a) 0, (b) 25, (c) 50, (d) 75, (e) 100 and (f) 150 $\mu\text{g/ml}$. ** $P < 0.01$ and *** $P < 0.001$.

wild-type cells. In addition, Figures 6a and b reveal that the cell migration and invasion ability recovered when cells overexpressed Axl. These results showed that BP exerts a strong effect on the viability, invasion and migration of human GBM cells by mediating Axl expression.

To further explore the role of Axl in the EMT process, we assessed changes in the gene expression profile and protein expression profile upon Axl overexpression induced using the plasmid pCDNA3.0-Axl. In the wild-type pCDNA3.0-neo group, we observed that EMT-related factors were downregulated by BP (Figures 7a and c). When Axl was overexpressed in cells, the expression of EMT-related markers reversed (Figures 7b and d).

The data demonstrated that BP reversed the EMT in GBM cells mediated by Axl regulation.

MAPK/ extracellular signal-regulated kinases (ERK) signalling pathway is involved in BP-mediated Axl regulation
Gas 6/Axl signaling promotes growth, survival, and proliferation is activated by MAPK/ERK signaling pathway which also known as the Ras-Raf-MEK-ERK pathway.¹⁰ We performed western blotting to examine the activation status of ERK and Akt during BP-induced pathway signalling in DBTRG cells. Strong activation of ERK was observed 1 h after BP exposure. To investigate the signalling

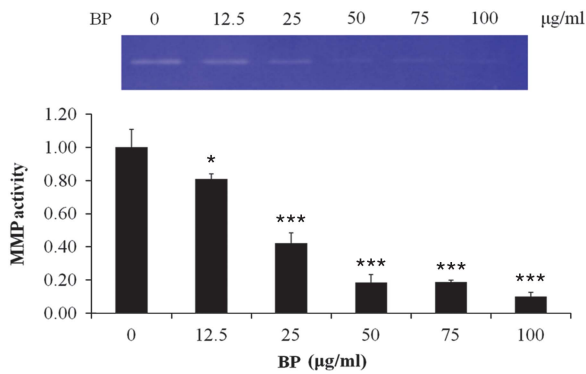


Figure 3. BP inhibits MMP activity in a dose-dependent manner in the GBM cell line DBTRG-05MG. The zymography assay results demonstrate that BP reduced the activity of MMPs in a dose-dependent manner in 24-h treatment, and the quantification is on the below. * $P < 0.05$, and *** $P < 0.001$.

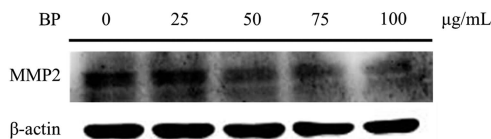


Figure 4. BP inhibits MMP2 expression in a dose-dependent manner in the GBM cell line DBTRG-05MG. MMP2 expression of DBTRG brain tumour cells treated with BP at 0, 25, 50, 75, 100 µg/ml, analysed after 24 h.

pathways critical for the BP induction of Axl expression, we used the chemical inhibitor PD98059 of ERK pathways to examine the requirement for activating each pathway in BP-mediated Axl downregulation. The inhibition of ERK kinase by PD98059 effectively blocked the BP-mediated inhibition of Axl. These results indicate that the ERK pathway is more likely to contribute to BP-induced regulation of Axl.

BP can inhibit tumour growth and reduce Axl and MMP2 expression in a xenograft model

This animal study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of China Medical University. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimise pain.

To evaluate the antitumour activity of BP *in vivo*, human brain cancer xenografts were established through SC injection of $\sim 2 \times 10^6$ DBTRG-05MG cells into the flank of nude mice. After the tumour size reached 100–250 mm³, mice were randomised into control and treatment groups ($n = 6$) and administered a daily SC injection of BP at 0, 300 or 500 mg/kg for 5 successive days and then a SC injection of BP once per 2 days for 1 month. As shown in Figure 9a, treatment with BP at 300 and 500 mg/kg significantly inhibited tumour growth. The GBM tumour tissues treated with BP exhibited downregulation of Axl and MMP2 expression after treatment, as determined through immunohistochemical staining. In this study of SC tumour implantation, the results indicated that BP not only inhibits tumour growth (Figure 9a) but also suppresses tumour invasion in parallel with a decrease of Axl and MMP2 expression in a dose-dependent manner (Figure 9b).

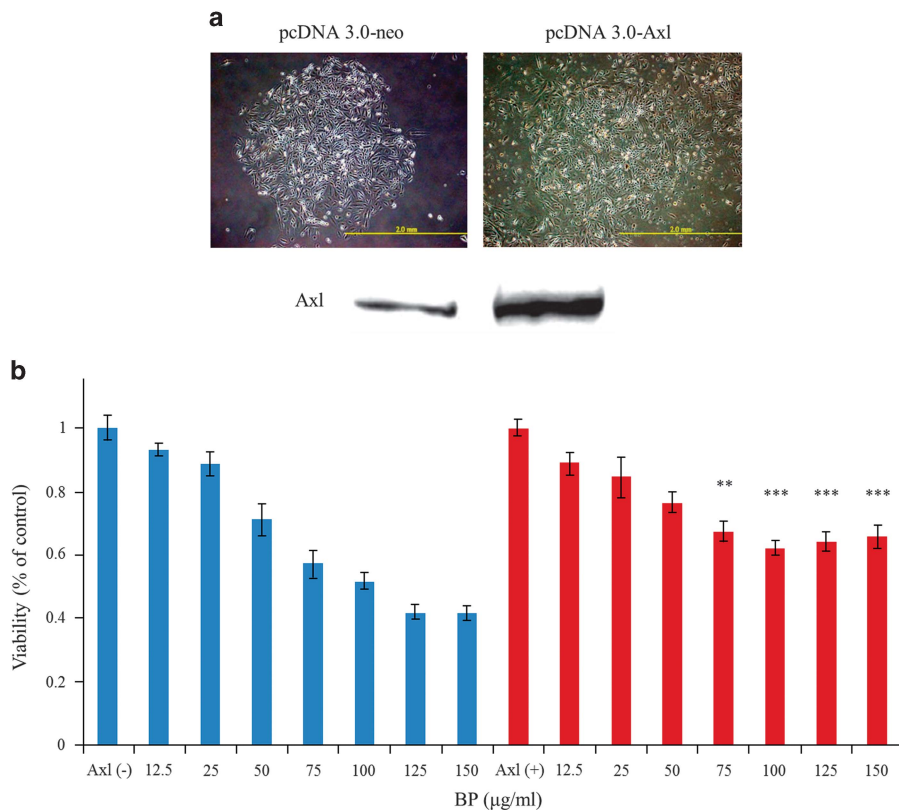


Figure 5. Cell viability was reversed by Axl overexpression in DBTRG cells. (a) The selection of colony of DBTRG cells transfected with pcDNA3.0-neo and pcDNA3.0-Axl. (b) The cell viability of DBTRG-neo and DBTRG-Axl with BP administered at various doses in 24-h treatment. ** $P < 0.01$ and *** $P < 0.001$.

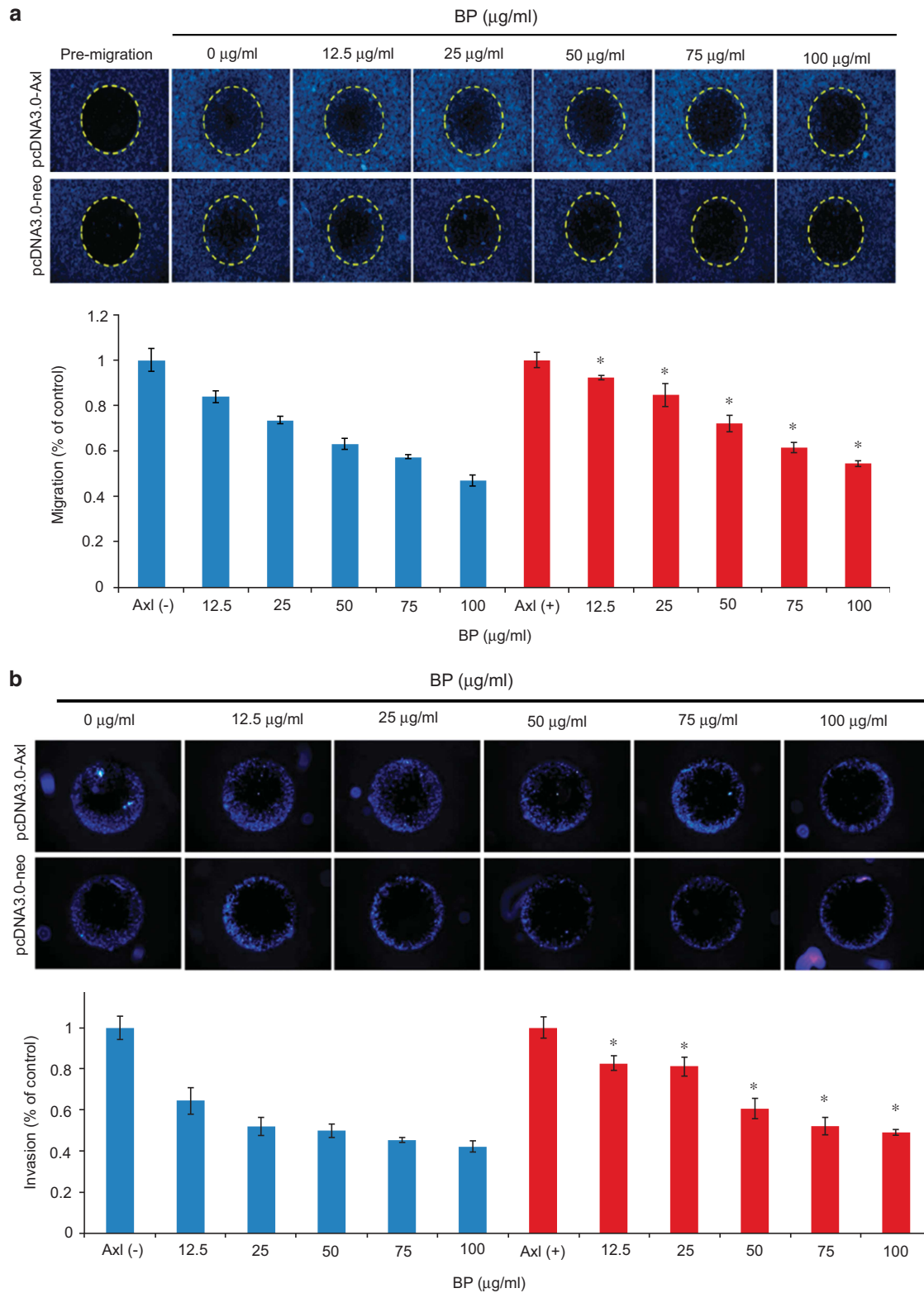


Figure 6. Cell migration and invasion were reversed by Axl overexpression in DBTRG cells. **(a)** Migration assay of DBTRG-neo and DBTRG-Axl cells performed using the Oris system, with BP administered at various doses in 24-h treatment. **(b)** Invasion assay of DBTRG-neo and DBTRG-Axl cells with BP administered in a dose-dependent manner in 24-h treatment. * $P < 0.05$.

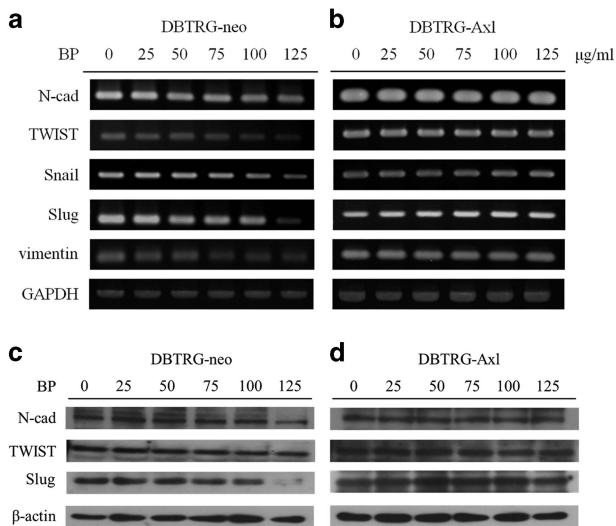


Figure 7. BP inhibits expression of EMT genes. Reverse transcriptase-PCR and western blot analysis of EMT-related genes in GBM cells administered BP at various doses in 24-h treatment. Downregulation of EMT-related genes. The results suggest that EMT was inhibited by BP in GBM cells (a and c). In addition, when Axl was overexpressed in DBTRG cells (DBTRG-Axl), EMT gene expression was recovered (b and d). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 1. Survival analysis and mean survival and median survival in four groups

	Mean survival	Median survival
Control wafer	21.83 ± 4.12	22
Gliadel wafer	32.17 ± 8.26*	29
10% BP wafer	27.43 ± 7.21	27
15% BP wafer	43.75 ± 10.61**	45
25% BP wafer	78.56 ± 25.25**	55

Abbreviation: BP, *n*-butylideneephthalide. * $P < 0.05$, ** $P < 0.01$.

BP prolonged the survival of rats with intracerebral tumours and reduced the tumour invasion size correlated with Axl expression. In 2011, we developed a local interstitial method for delivering BP by using polymer wafers²⁵ and designated the wafers as BP wafers. Local delivery of drugs by using controlled-release polymers can increase the concentration of chemotherapeutic agents in the brain and improve retention time. Controlled-release polymers bypass the blood-brain barrier (BBB), preventing systemic toxicity. We used an orthotic model to evaluate whether this BP wafer could prolong the survival of rats and inhibit GBM cell invasion in brain tissue. After surgery, we observed the activity of the rats and used Kaplan–Meier survival curves to evaluate their vital signs. The relative survival rate of the rats is shown in Figure 9c and Table 1; the group of 25% BP wafer prolonged survival 3.7-fold compared with the control group ($P < 0.01$); the group of 25% BP wafer prolonged survival 2.4-fold compared with the Gliadel wafer group. The brain tissues treated with BP wafers showed that invasive cells decreased in a dose-dependent manner and that Axl expression was downregulated (Figure 9d).

These data indicated that the small molecule targeting Axl, BP, inhibits brain tumour migration and invasion *in vivo*.

DISCUSSION

In our previous studies, we investigated Axl downregulation and demonstrated that BP has high potential to be used as an anticancer drug in GBM brain tumours.^{19–25} In the current study, we investigated whether BP mediation of Axl is involved in GBM cell migration and invasion. First, Axl mRNA and protein regulation after BP treatment in a time- and dose-dependent manner were evaluated in 2 DBTRG cell lines (Figure 1); in addition, we studied the cell migration and invasion ability (Figure 2). The results showed that BP mediation of Axl mRNA expression decreased 12 h after BP treatment, with the expression being the lowest after 24 h. The Axl protein expression was consistent with downregulation after 24 h of BP dose-dependent treatment. In addition, the results showed that migrating and invasive cells were repressed in a dose-dependent manner after BP treatment for 24 h (Figure 2). As shown in Figures 1 and 2, the decreasing migration and invasion capacity was parallel with the downregulation of Axl-1 by BP. This result implicates that the inhibition of tumour invasion by BP might be caused by Axl. To confirm that Axl downregulation causes the inhibition of tumour invasion, we induced Axl overexpression by transfecting cells with the pcDNA3.0-Axl plasmid and then assessed the role of Axl in cell proliferation, migration and invasion. We determined that BP significantly reduced the viability, invasion and migration of human GBM cells by mediating Axl RTK expression (Figures 5 and 6; $P < 0.05$). This suggests that Axl has a key role in the inhibition of GBM invasion and migration by BP. MMPs have a crucial role in tumour invasion and metastasis, and are essential components for degrading the ECM; therefore, we investigated the change of MMP expression after BP treatment. We observed significant changes in the expression levels of several MMPs induced by BP (Figures 3 and 4), providing additional evidence that BP inhibits tumour invasion and migration.

Because the EMT provides tumour cells with enhanced migratory and survival attributes that facilitate malignant progression, we demonstrated that BP inhibited the EMT by reducing N-cadherin, Twist, Snail and Slug expression, indicating that BP can reverse the EMT and, thus, inhibit tumour invasion. Furthermore, to determine the role of BP in Axl and EMT regulation, we determined that the reversal of the EMT by BP is abrogated by the tumour cell overexpression of Axl, which is accompanied by the restoration of N-cadherin, Twist and Slug expression (Figure 7). The results suggest that BP affecting EMT plasticity might be related to Axl regulation.

In 2010, Gjerdrum stated that Axl is an essential EMT-induced regulator in breast cancer, and Axl expression is significantly associated with reduced patient survival.¹⁷ Because the author sought to determine the activation of which EMT programme transcription factor leads to Axl upregulation, they analysed a nonmalignant breast epithelial cell line (MCF10a) transduced with retroviral constructs that expressed Twist, Zeb2, Snail or Slug. The results showed that EMT induction by these transcription factors was accompanied by enhanced Axl expression. In 2014, another study stated that Axl induces the EMT and regulates the function of breast cancer stem cells.¹⁸ However, no study has reported that small molecules affect the EMT via Axl. Our study is the first to report that small molecules affect the EMT status in GBM via Axl. This finding can be used to develop new drugs for repressing GBM invasion.

In this study, we used western blot analysis to determine the signalling pathway involved in BP-mediated Axl gene expression. As shown in Figure 8a, ERK was significantly phosphorylated after BP treatment. We then used the ERK-specific inhibitor PD98059 to examine whether the mediation of Axl by BP involved ERK signalling. As shown in Figure 8b, BP-mediated Axl expression was decreased when cells were pretreated with the ERK inhibitor. This demonstrated that BP regulates Axl through the mitogen-

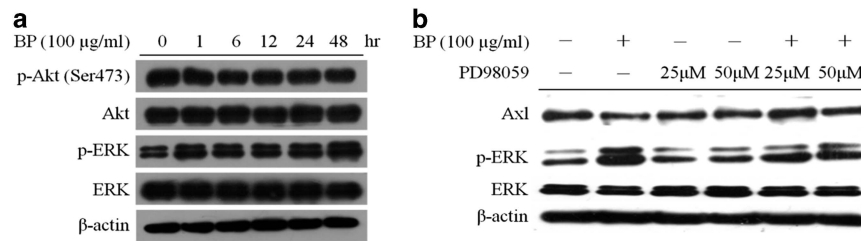


Figure 8. Signalling pathways involved in BP-mediated Axl repression. **(a)** DBTRG cells were treated with BP for 0–48 h. Western blot analysis was performed for p-Akt, Akt, p-ERK and ERK. β-Actin was used as an internal control. **(b)** DBTRG cells were pretreated with the ERK inhibitor PD98059 (25 and 50 µM) for 1 h. Western blot analysis was performed for p-ERK, ERK and Axl expression. β-Actin was used as an internal control.

activated protein kinase/ERK pathway. In 2008, we determined that Nur77 is another potential target of BP chemotherapy in GBM.¹⁹ However, the upregulation of Nur77 occurs via a PKC signalling pathway, mediating the antitumour activity of BP in brain tumour cells. Therefore, we propose that BP regulation of various target genes might occur via different pathways. For example, taxol was found that combined inhibition of Notch and HER2 signalling pathways can indeed decrease recurrence rates for breast cancers that are characterized by HER2 overexpression. On the other hand, regarding to the chemoresistance on taxol, Toll-like receptor-4 is more relevant that could be mediated by activation of the nuclear factor-κB pathway^{26,27} This supports our proposal that the same drug with different target genes mediates different pathways.

Finally, to confirm whether the *in vitro* mechanisms are the same as those *in vivo*, we used two tumour animal models for our study. First, in the xenograft study, we demonstrated that BP not only inhibited tumour growth (Figure 9a) but also suppressed Axl and MMP2 expression in a dose-dependent manner (Figure 9b). Furthermore, in the orthotopic tumour implant study, we used a biodegradable polymer (CPP-SA) carrying BP to treat implanted tumours. We observed that this treatment not only significantly prolonged the survival of rats (Figure 9c, Gliadel wafer $P < 0.05$, 15% BP wafer $P < 0.01$, 25% BP wafer $P < 0.01$) but also that the number of invasive cells decreased after BP treatment in a dose-dependent manner (Figure 9d). Local delivery of drugs by using controlled-release polymers could extend the drug release time and enhance the local drug concentration. In the case of Gliadel wafer, they tracked the BCNU diffusion distance by measuring the radioactivity in a rabbit brain tissue section. BCNU diffused only 2 mm and rapidly accompanying a concentration decrease 72 h after implantation, which may be the reason the residual tumour cell was not eradicated and the patients' survival cannot prolong more. We used an orthotopic tumour implant model to demonstrate that our BP wafer diffused over a distance of more than 20 mm in dog brains, and the released BP concentration (3.03 mM) showed a higher sustenance than did the IC₅₀ (100 µM) 120 h after BP wafer implantation (data not shown). It showed that our BP wafer could diffuse long distance and sustain in effective dose. Moreover, Holland²⁸ showed that brain tumour tissue cells migrate through the normal parenchyma, accumulate immediately below the pial margin (subpial spread), surround neurons and vessels (perineuronal and perivascular satellitosis), and migrate through the white matter tracks (intrafascicular spread). The ultimate result of this invasive behaviour is the spread of individual tumour cells and diffusion over long distances and into regions of the brain essential for the poor survival of the patient.²⁸ On the basis of this reason, we developed a novel small-molecule delivery system that could diffuse for a long distance and could reduce GBM cell migration and invasion.

Vajkoczy *et al.* described the identification, functional manipulation, *in vitro* and *in vivo* validation and preclinical therapeutic

inhibition of a target RTK mediating glioma growth and invasion.²⁹ We plan to investigate whether the invasion capacity is related to Axl expression and rat survival in an intracranial tumour implant BP wafer model. Our results regarding rat survival indicated that the BP wafer reduced GBM cell invasion by dose dependently suppressing Axl expression.

In summary, we demonstrated that a novel small molecule, BP, inhibited tumour migration and invasion by downregulating Axl and, thus, reduced EMT. In addition, a biodegradable interstitial release polymer loading BP exhibited an extended diffusion distance and a prolonged release time, significantly prolonging animal survival. This may contribute a potential therapeutic target for clinical intervention.

MATERIALS AND METHODS

Chemicals and reagents

BP was purchased from Alfa Aesar (Ward Hill, MA, USA). The cell culture medium RPMI-1640 and fetal bovine serum were purchased from Hyclone (Logan, UT, USA). Dimethylsulphoxide, MTT and β-actin were purchased from Sigma-Aldrich (St Louis, MO, USA). An RNeasy Midi Kit and Omniscript RT Kit were purchased from Qiagen (Valencia, CA, USA). Lipofectamine 2000 and Geneticin (G418) were purchased from Invitrogen (Carlsbad, CA, USA). Antibody of Axl is from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Genetex (Irvine, CA, USA). MMP2, Akt, p-Akt, Erk, p-Erk are from Cell Signaling (Beverly, MA, USA). The Horseradish peroxidase-linked IgG secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Cell lines and cell culture

The human GBM cell line DBTRG-05MG (BCRC 60380) was obtained from the Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan). The cells were maintained using the RPMI-1640 (Hyclone) medium containing 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell cytotoxicity assay

The cell viability was evaluated using the MTT assay. Cells were seeded in 96-well plates (5×10^3 cells/well) containing 200 µl of a growth medium. The BP doses were serially diluted in various concentrations (0, 25, 50, 75, 100 and 125 µg/ml). After 24 h, the drug-containing medium was removed, and a fresh medium containing 500 µg/ml MTT was added. The absorbance of the dissolved solutions was determined using a PowerWave Microplate ELISA Reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 570 nm. The results were determined through three independent experiments.

Western blot analysis

Approximately 1×10^6 cells were cultured in 10-cm dishes and then incubated in various concentrations of BP. The cells were lysed on ice with 100 µl of PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc., Kyunggi-do, Korea) and centrifuged at 13 000 r.p.m. for 15 min at 4 °C. The protein concentrations in the supernatants were quantified using a BSA protein assay kit (Novagen, Darmstadt, Germany). Electrophoresis was

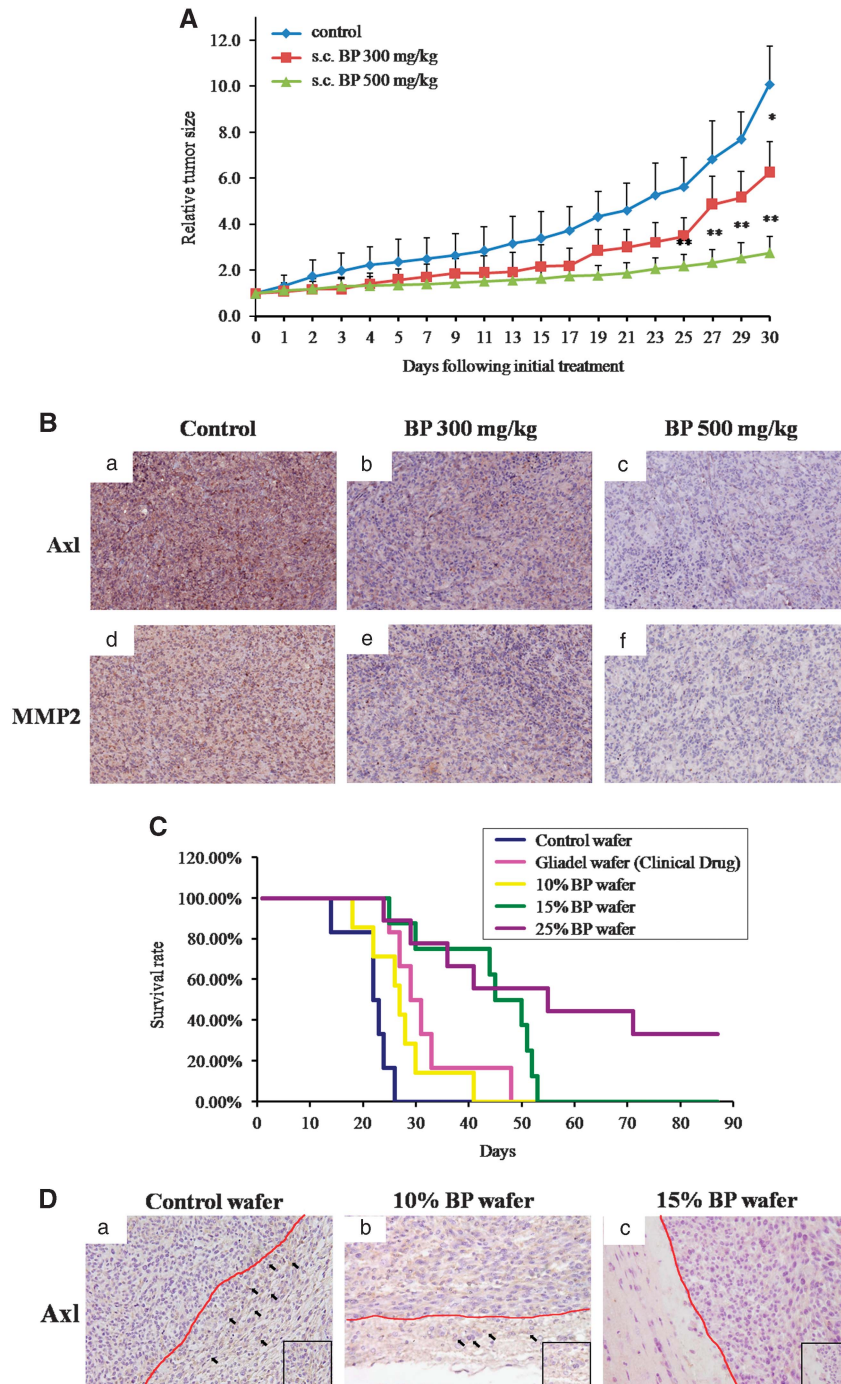


Figure 9. *In vivo* xenografts and orthotopic animal study demonstrated that BP inhibited tumour growth and suppressed Axl and MMP2 expression *in vivo*. **(a)** Tumour sizes were measured using calipers and were monitored until day 30. **(b)** Tissue sections stained for Axl and MMP9 in the xenograft model. **(c)** Rat survival rate in the orthotopic animal study. **(d)** Brain tissue stain for Axl photographed under a light microscope at a magnification of $\times 200$.

performed on a polyacrylamide gel electrophoresis by using 50 μg of reduced protein extract per lane. Resolved proteins were then transferred to polyvinylidene difluoride membranes. Filters were blocked with 5% nonfat milk overnight and then incubated with 1:1000 dilutions of primary antibodies for 16–18 h at 4 $^{\circ}\text{C}$. Membranes were washed three times with 0.1% Tween-20 and incubated with a 1:2000 dilution of an HRP-conjugated secondary antibody for 1 h at room temperature. The results were detected using the Western Lightning Chemiluminescence Reagent Plus (Advanta, Menlo Park, CA, USA) and quantified using a densitometer.

Reverse transcriptase–PCR

Total RNA from each sample was isolated using an RNeasy Midi Kit and RNase-free DNase Set (Qiagen) according to the manufacturer's protocols. The concentration was calculated spectrophotometrically, and the concentration of RNA was adjusted to 1 $\mu\text{g}/\mu\text{l}$. 1 μg of total RNA from each sample was used to generate cDNA by using the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. The PCR thermal cycling profile comprised an initial denaturation step at 94 $^{\circ}\text{C}$ for 5 min, 30 cycles of 40 s of denaturation at 94 $^{\circ}\text{C}$, 50 s of annealing at 56 $^{\circ}\text{C}$, 1 min of

Table 2. Gene-specific primer sequences for reverse transcription-PCR

Gene symbol	Primer sequences	Annealing temperature (°C)	Cycles
Axl (F)	5'-GGTGGCTGTGAAGACGATGA-3'	56	30
Axl (R)	5'-CTCAGATACTCCATGCCACT-3'		
N-cad (F)	5'-CAACTTGCAGAAAACCTCCAG G-3'	57	30
N-cad (R)	5'-ATGAAACCGGGCTATCTGCTC-3'		
Twist (F)	5'-GGGAGTCCGACGTCTTACGA-3'	55	30
Twist (R)	5'-AGACCGAGAAGGCGTAGCTG-3'		
Snail (F)	5'-GCTGCCAATGCTCATCTGGGACTCT-3'	60	30
Snail (R)	5'-TTGAAGGGCTTTCGAGCCTGGAGAT-3'		
Slug (F)	5'-AGATGCATATTCGGACCCAC-3'	57	30
Slug (R)	5'-CCTCATGTTTGTGACAGGAGA-3'		
Vimentin (F)	5'-GCAATCTTTCAGACAGGATGTTGAC-3'	55	30
Vimentin (R)	5'-GATTTCCTCTTCGTGGAGTTTCTC-3'		
GAPDH (F)	5'-TGAAGGTCCGGAGTCAACGGATTGGT-3'	56	30
GAPDH (R)	5'-CATGTGGCCATGAGGTCCACCAC-3'		

extension at 72 °C and a final 5 min extension step at 72 °C, then finally keep at 4 °C. The name and sequences of the primers, cycles and annealing temperature for each pair are listed in Table 2. The PCR products were separated on 1 or 2% agarose gels, stained with ethidium bromide and visualised using the Vilber Lourmat imaging system (Marne la Vallee, France), and the level of glyceraldehyde 3-phosphate dehydrogenase was used as the control.

In vitro transfection

The plasmid was provided by the Shuang-En Chuang, Associate Investigator, National Health Research Institutes. To create an Axl-overexpressing DBTRG-05MG cell line, DBTRG-05MG cells were transfected with a 4- μ g pcDNA3.0-Axl or pcDNA3.0-neo vector by using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were subjected to selection for stable integrants through exposure for 3 weeks to 200–400 μ g/ml G418 (Invitrogen) in a complete medium containing 10% fetal bovine serum. The cells were then assessed for the overexpression of Axl by using western blot analysis.

Wound-healing assays

DBTRG-05MG cells were seeded in 6-well plates and cultured until nearly confluent (~90%). The cells were then incubated in RPMI-1640 medium supplemented with 25 μ g/ml mitomycin C pretreated for 30 min. A wound was then created by manually scraping the cell monolayer with a 200- μ l pipette tip. The cultures were washed twice with phosphate-buffered saline to remove floating cells. Cell migration into the wound was observed and recorded at two preselected time points (0 and 24 h) in eight randomly selected microscopic fields for each condition and time point. The distance travelled by the cells was determined by measuring the wound width at various time points and then subtracting it from the wound width at time zero. The values were expressed as the migration percentage, with the gap width at 0 h set as 0%.

In vitro migration assay and invasion assay

Cells (2×10^4) were planted on the top of polycarbonate Transwell filters (with or without Matrigel (BD Bioscience, Bedford, MA, USA) for Transwell assay). The BD Matrigel matrix is composed of laminin, collagen IV, nidogen/entactin and proteoglycan on polyethylene terephthalate membranes containing 8- μ m pores. For Transwell migration assays, cells were suspended in a medium without serum, and this medium was used in the bottom chamber. For the invasion assay, cells were suspended in a medium without serum and a medium supplemented with serum in the bottom chamber. The cells were incubated at 37 °C for 24 h. The nonmigratory or noninvasive cells in the top chambers were removed with cotton swabs. The cells that invaded or migrated through the membrane were fixed in 100% methanol for 10 min, were then stained with haematoxylin and counted under a microscope. Each experiment was repeated three times.

In addition, we used an Oris system to evaluate cell migration and invasion. The cells were seeded at 1×10^4 cells per well into the 96-well plate of an Oris Cell Invasion Assay Kit (Pla-typus, Madison, WI, USA). The

plate was incubated for at least 16 h at 37 °C. The stoppers were then removed. A collagen I overlay was added to create a three-dimensional ECM environment for invasion and incubated for 1 h at 37 °C. A cell culture medium containing various concentration of BP was added, the cells were allowed to migrate or invade for 24 h, and the cells were stained with Hoechst 33342 (Invitrogen) before images were captured.

Zymography for MMP activity

DBTRG cells were plated in six-well plates and added to a 1.5-ml medium containing various concentrations of BP. After 24 h of treatment, the cell-conditioned medium was collected and analysed for MMP activity by using gelatin zymography. Our focus was on MMP2, because this is the major gelatinase that is expressed by glioma cell lines. The samples were mixed with an equal volume of a 2 \times sample buffer (0.005% Bromophenol Blue, 20% glycerol, 4% SDS and 100 mM Tris-Cl (pH 6.8)) and subjected to nonreducing electrophoresis in 10% polyacrylamide gels containing gelatin (1 mg/ml). The gels were then washed with 2.5% Triton X-100 and incubated in a digestion buffer at 37 °C for 48 h. The gels were stained with Coomassie blue and subsequently destained.

Polymer preparation

SA prepolymer preparation. SA was recrystallised two times in alcohol. SA monomers (2.7 g) were refluxed with 60 ml of excess acetic anhydride for 30 min at 135–140 °C in a vacuum (10^{-4} torr). Excess unreacted acetic anhydride was removed, and the SA prepolymer was dried through evaporation in a 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 of excess acetic anhydride for 30 min at 150 °C in a vacuum (10^{-4} torr). After cooling, the solution was filtered using filter paper. Excess unreacted acetic anhydride was removed, and the CPP prepolymer was recrystallised 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. Dry ether (dimethylformamide/dry ether, 1:9) was then added and incubated overnight. The dry ether and dimethylformamide were removed, and the CPP prepolymer crystals were dried in a vacuum.

Poly (CPP-SA) copolymer preparation. A mixture of CPP and SA prepolymers at a ratio of 20:80 was added to a glass bottle in a vacuum. The CPP and SA prepolymers were heated at 180 °C in an oil bath for 1.5 h, and the pressure was reduced to 10^{-4} torr. Throughout the polymerisation, the vacuum pressure was reduced every 15 min. After 1.5 h, poly(CPP-SA) copolymers were washed with dichloromethane, and petroleum ether was then added to precipitate the poly(CPP-SA) copolymers. Finally, the poly(CPP-SA) copolymers were washed with anhydrous ether and dried in a vacuum. The p(CPP:SA; 20:80) polymers containing BP (BP wafer) or BCNU (BCNU wafer) were synthesised according to the method described by Domb and Langer.³⁰

Antitumour activity in vivo

Xenograft model. Xenograft mice were used as a model to study the cytotoxicity of BP *in vivo*: the procedure for implanting cancer cells was similar to that used in previous studies. Male congenital athymic BALB/c nude (nu/nu) mice were purchased from the National Science Council (Taipei, Taiwan), and all procedures were performed in compliance with the standard operating procedures of the Laboratory Animal Center of China Medical University Hospital (Taichung, Taiwan). All experiments were conducted using 6- to 8-week-old mice weighing 18–22 g. The backs of the mice were subcutaneously implanted with 2×10^6 DBTRG-05MG cells. When the tumours reached 100–250 mm³ in volume, the mice were divided randomly into control and test groups consisting of six mice per group. The BP treatment group was administered a SC injection at 300 or 500 mg/kg daily for an initial 5 days. After 5 days, the treatment frequency was changed to once per 2 days. All mice used for therapy response evaluations were killed 1 month after treatment in a double-blinded manner. The control group was treated only with a vehicle. The mice were

weighed once every 3 days until day 30 to monitor the effects; simultaneously, the tumour volume was determined by measuring the length (*L*) and width (*W*). The tumour volume at day *n* (TV_{*n*}) was calculated as TV (mm³) = (*LW*²)/2.

Orthotic model. Male F344 rats (230–260 g) 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 China Medical University (Taichung, Taiwan). The rat glioblastoma tumour line 9L was obtained from the BCRC. For intracranial implantation, 9L glioblastoma fragments measuring ~2×2×1 mm³ were excised at the time of implantation. Using a microsurgical technique, a midline incision was made on the posterior aspect of the head and the periosteum was swept laterally to expose the sagittal, coronal and lambdoidal sutures. A 3-mm burr hole was made over the left parietal region with its centre 5–6 mm behind the coronal suture and 3–4 mm lateral to the sagittal suture. A cruciate incision was made on the dura. On the 7th day after tumour implantation, the rats were randomly divided into five groups (*n* ≥ 6) for initiation of treatment and subjected to another operation for the insertion of polymeric discs with double blind. The BP wafers were prepared using the procedures described by Domb and Langer.³⁰ The animals were killed when they exhibited an ataxic gait, or, in the advanced stages, when they displayed extensor posturing of the hind legs.

Immunohistochemical staining

The tumour tissues were fixed in 10% formalin overnight and then embedded in paraffin. Paraffin sections (5 μm) were deparaffinised in xylene and rehydrated through a graded series of (75–100%) ethanol solutions. The sections were incubated for 10 min in hydrogen peroxide and protein blocks at room temperature, followed by 1 h of incubation with a dilution primary antibody in phosphate-buffered saline. Subsequently, we used a detection system (UltraVision Quanto Detection System, Thermo Scientific, Waltham, MA, USA) to detect the signal. Finally, sections were counterstained with haematoxylin, mounted, observed under a light microscope at a magnification of ×200 and photographed.

Statistical analysis

The experiment groups and size were decided after discussion with statistic expert in our institute. And all experiments were performed in three or more independent assays, which yielded highly comparable results. Data are summarised as the mean ± standard deviation. Statistical analysis of the results was performed using a Student's *t*-test for unpaired samples. The *P* value < 0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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