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Research paper

Evaluation of in vitro and in vivo antitumor activity of BCNU-loaded PLGA wafer against 9L gliosarcoma

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Abstract

The purpose of the present study was to develop implantable BCNU-loaded poly(D,L-lactide-co-glycolide) (PLGA) wafer for the controlled release of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and to evaluate its in vitro and in vivo antitumor activity. The release rate of BCNU from PLGA wafer increased with the increase of BCNU amount loaded and the release was continued until 7 days. In vitro and in vivo antitumor activity of BCNU-loaded PLGA wafer was investigated using in vitro cytotoxicity against 9L gliosarcoma cells and a subcutaneous (s.c.) solid tumor model of 9L gliosarcoma, respectively. The wafers containing BCNU showed more effective cytotoxicity than BCNU powder due to its short half-life and inhibited the proliferation of 9L gliosarcoma cells. BCNU-loaded PLGA wafer delayed the growth of the tumors significantly and increasing the dose of BCNU in the wafer resulted in a substantial regression of the tumor. These results of antitumor activity of BCNU-loaded PLGA wafer demonstrate the feasibility of the wafers for clinical application.

Keywords: Malignant gliomas; BCNU; PLGA wafer; 9L gliosarcoma; Antitumor activity

1. Introduction

Malignant gliomas account for 13–22% of brain tumors and the median survival of the patients is less than 1 year [1]. Even though patients suffering from glioblastoma take surgical resection, external-beam radiotherapy, and systemic chemotherapy. In other words, the conventional therapies cannot extend the median survival of patients significantly because these tumors tend to recur within 2 cm of the treated site [2]. In recent years, some efforts in order to improve median survival rate of patients harboring malignant gliomas have concentrated on locally implantable drug–polymer devices for obviating the need of drug penetration to blood–brain barrier (BBB) [3,4]. Implantable polymers can facilitate release of chemotherapeutic agents adjacent to the tumor site. Moreover, their advantages are not only achievement of very high local concentrations of anticancer agents but also minimization of systemic toxicity due to circumvention of the requirement for the anticancer agent to cross the BBB. Non-biodegradable polymers as an EVAc [5] have been used for polymeric drug delivery devices implanted into the brain. The localized, controlled delivery of anticancer agents using biodegradable polymeric implant has also been developed as an alternative to the systemic administration of chemotherapeutic agents for treating malignant gliomas [6,7].

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, also called as carmustine) is one of the most widely used antineoplastic agents for the treatment of malignant gliomas [8]. It is appropriate for anticancer agent to penetrate BBB because of its good lipid solubility and low molecular weight [9]. However, therapeutic efficacy of BCNU was limited because of its short half-life in vivo as well as in vitro.

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Synthetic biodegradable polymers, such as aliphatic polyesters and polyanhydrides, have been extensively studied for implantable microspheres or monolithic devices [10,11]. Among the synthetic biodegradable polymers, poly(D,L-lactide-co-glycolide) (PLGA) is a biologically inert polymer that is capable of releasing drug in a diffusion-regulated, controlled, and hydrolyzed fashion [12,13]. Drug release period from the PLGA matrix can be modulated from days to years by varying degradation period of PLGA [14]. PLGA provides the additive advantages of being fully biodegradable and subsequent obviating the problems associated with permanent implants based on non-biodegradable polymers [15,16]. Especially, the biocompatibility of PLGA was proven in the brain of rodents and human [17,18].

To date, one of the most outstanding results is BCNU-loaded polyanhydride wafer, which recently won approval from FDA adjunct therapy for treatment of brain tumors [19,20]. Clinical trials with this controlled delivery polymer, Gliadel®, have shown an increase of approximate 3 months in median survival rate of patients, all of whom had failed prior therapy [21]. In our previous study [22,23], BCNU-loaded PLGA microparticles were prepared by spray-drying method for long term delivery of BCNU. However, there was lots of the drug loss originated from the use of organic solvent in spray-drying method. In this study, we fabricated wafers by compression molding of mixtures of BCNU and PLGA for long term delivery of BCNU without using organic solvent. The release profiles of BCNU from PLGA wafers were studied. In vitro antitumor activity of BCNU released from PLGA wafers with the different loading amount of drug against 9L gliosarcoma cells was assessed. For the in vivo antitumor activity test, the tumor was established on the flank of rats. The BCNU-loaded PLGA wafers were implanted adjacent to the tumor and their antitumor activities were assessed with tumor volume change of the animals.

2. Materials and methods

2.1. Materials

BCNU was purchased from Sigma Chemical Co. (St Louis, MO, USA) and stored at −20 °C until use. PLGA (50:50 mole ratio of lactide to glycolide,) having molecular weight of 8000 g/mole (Resomer®, RG 502H) was purchased from Boehringer Ingelheim (Germany). Methylene chloride (MC, Tedia, Japan) and methanol (Junsei, Japan) were used as received. All other chemicals were reagent grade. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), antibiotics (10 μg/ml of amphotericin and 10 U/ml of penicillin G sodium), TRIZMA®, and trypsin/1,2-ethylenediaminetetraacetic (trypsin-EDTA) were purchased from Gibco BRL® (Grand Island, NY, USA). Sulforhodamine B (SRB) and tricholoroacetic acid (TCA) were also purchased from Sigma Chemical Co. (St Louis, MO, USA). T-25, T-75, 6-well, and 96-well plates were purchased from Falcon Co. (Lincoln Park, NJ, USA).

2.2. Preparation of BCNU-loaded PLGA wafers

BCNU and PLGA were mixed by vortex-mixing. Twenty-five milligram of mixtures of BCNU and PLGA were compression molded into wafers using Carver Press (MH-50Y CAP 50 tons, Japan) at 20 kgf/cm^2 for 5 s at room temperature. The wafers were 5 mm (diameter) × 1 mm (thickness) in size with a flat surface and stored at 0 °C until use.

2.3. In vitro release study

BCNU in PLGA wafer was assayed by HPLC in order to determine the drug content in the wafer. BCNU-loaded PLGA wafers were dissolved in 2 ml of methylene chloride. PLGA was precipitated by adding 18 ml of methanol and after centrifugation, 100 μl of aliquots of supernatant were analyzed by HPLC. All samples were analyzed using HPLC system equipped with a Model P-2000 pump, a Model AS-3000 autosampler, and a Model UV-1000 UV detector at 237 nm (Thermo Separation Products, Fermont, CA). The column used was μ-Bondapak™ C18 (3.9×300 mm^2, Waters, Milford, MA). Mobile phase was deionized water/ methanol (6:14 v/v) mixture and flow rate was adjusted 1.0 ml/min.

Release of BCNU from PLGA wafer was monitored for a period of incubation in PBS, pH 7.4 at 37 °C. Wafers were individually placed in 20 ml of PBS with constant shaking at 110 rpm. At specific time following incubation wafers were retrieved and freeze-dried for 48 h. After precipitation of PLGA with methanol, 20 μl of aliquots of supernatant were analyzed by HPLC. The amount of BCNU released from the wafer was calculated by the amount of BCNU remained in the wafer after specific release test period due to the instability of BCNU in the release test condition.

2.4. Cell culture

9L gliosarcoma was provided by Korean Cell Line Bank. The cells were grown in DMEM medium was added antibiotics (10 μg/ml of amphotericin and 10 U/ml of penicillin G sodium) and supplemented with 10% (v/v) FBS. The cells were cultured in a 95% air/5% CO_2 atmosphere at 37 °C in a humidified incubator, and were dissociated with 0.05% trypsin-EDTA in case of transferring or dispensing before experiment.

2.5. Cytotoxcity test

The experimental cells were plated on 6-well flat-bottomed plates with a density of 1×10^5 cells/well and incubated for 24 h at 37 °C in the CO_2 incubator. The cells were counted in a hemacytometer (Reichert Co., U.S.A) using the trypan blue exclusion method. After the cells were attached on 6-well flat-bottomed plates, the BCNU powder was dissolved in cell culture medium at various concentrations (0.3, 1, 10, 30, and 100 μg/ml) and BCNU-loaded PLGA wafers containing a concentration of 3.85, 10, 20, and 30% were directly added to cell culture medium of 3 ml, respectively. The cells were incubated with the wafers for 3 days at 37 °C in the CO_2 incubator. And the cells were washed with PBS after removing the wafers and the culture medium. Removed wafers were dipped into the 6-well flat-bottomed plate and incubated the cells for 24 h at a density of 1×10^5 cells/well, repeatedly. Cell morphology was observed after 3 days and 6 days treatment. The cells were observed by microscopy (Nicon TE-300) and photographed by an attached camera (Nicon F70). The cytotoxicity following the above-mentioned treatment was evaluated by the SRB assay which measures whole culture protein content as an index of tumor cell viability. The SRB assay is an indirect measure of cell density or number of living cells attached to the culture plate. Briefly, the culture medium was aspirated and the cells were fixed with 10% cold TCA. The plates were incubated for 1 h at 4 °C and then washed several times with tap water to room temperature for 30 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed and cultures were rinsed several times with 1% (v/v) acetic acid to remove unbound dye. Residual wash solution was removed and the plates were air-dried. The bound dye was then solubilized with 2 ml of 10 mM unbuffered TRIZMA® base solution (pH 10–10.5). After solubilizing, the absorbance was measured with a SpectraMax 250 microplate reader (Molecular Devices, Menlo Park, CA, USA) at a wavelength of 520 nm. The results of two independent experiments were expressed as treatment over control (T/C) values; cell viability (%) = $T/C\times100$. All experiments were performed in triplicate.

2.6. Animal and anesthesia

Male Fisher rats weighing about 200 g of body weight were obtained from Charles River Japan Inc. (F 344/DuCrj; Kanagawa, Japan). The animals were anesthetized with an intraperitoneal (i.p.) injection of 2–4 ml/mg of a stock solution containing ketamin (90 mg/kg) and xylazine (10 mg/kg).

2.7. In vivo antitumor activity test

A solid tumor was established upon subcutaneous (s.c.) injection of 0.1 ml of the cell suspension (1×10^5 cells per rat) to the flank of male Fischer 344 rats. The established solid tumor was excised, sliced into pieces approximately 0.5 cm^3 and dissociated into smaller fragments in trocha. The solid tumor was inserted into other rats in every 2 weeks and this process was performed on 5 times. BCNU-loaded PLGA wafers were sterilized using UV sterilization. The wafers were put on FALCON® petri dish and both sides of the wafers were exposed to UV irradiation for 30 min in the clean bench prior to treatment. On the 10th day after the 5th passage of solid tumor, all the animals were operated for the treatment of the established tumor. Treatment was initiated when the tumors reached a volume of approximately 500 mm^3. The flank of the rat was shaved and prepared with 70% ethyl alcohol and povidone solution. Small incision was made above the tumor. Each 3.85, 10, 20, 30% BCNU-loaded PLGA wafer (4.8, 12.5, 25, and 37.5 mg/kg, respectively) was implanted next to the tumor and the wounds were sutured. Only PLGA wafer without BCNU was used as control. Tumor growth was documented by measuring the length and width of the tumors with dial calipers twice weekly. Tumor volume was calculated by formula for the approximation of the volume of a spheroid; Tumor Volume = $\text{Length}\times\text{Width}^2/2$ [24]. The serial measurement of tumor volume and body weight was followed until the day at which the animals were sacrificed.

3. Results and discussion

3.1. In vitro release study

BCNU is an effective chemotherapeutic agent used in the treatment of meningeal leukemia and primary and metastatic tumors of the brain. Generally, it is known that BCNU shows antitumor activity by alkylating DNA or RNA of tumor cells and has no interaction with other alkylating agents. Hydrolysis of BCNU is dependent on pH of solution [8]. It is the most stable at pH 4, however, it decomposes rapidly in acid and in solutions above pH 7. Therefore, BCNU has a half-life of 50 min in vitro and less than 15 min in vivo. It is also known that BCNU has low melting point of 30–32 °C and is very unstable to light.

Drug content in BCNU-loaded PLGA wafer prepared from the mixtures of BCNU and PLGA was from 95 to 105% and we could confirm that BCNU was dispersed in PLGA matrix. The release profiles of BCNU from BCNU-loaded PLGA wafers are shown in Fig. 1. The release rate of BCNU from PLGA wafer increased with the increase of BCNU loading amount and the period of 100% BCNU release was almost 7 days. After 6 h, it showed an initial burst. Initial burst also increased with the increase of BCNU loading amount. The reason of initial burst could be due to diffusional release of drug particles on the surface of wafers and a higher drug loading amount resulted in a high amounted drug particles on the surface of wafers. The release rate and pattern of drug from PLGA matrix is mainly dependent not only on diffusion of drug through the matrix

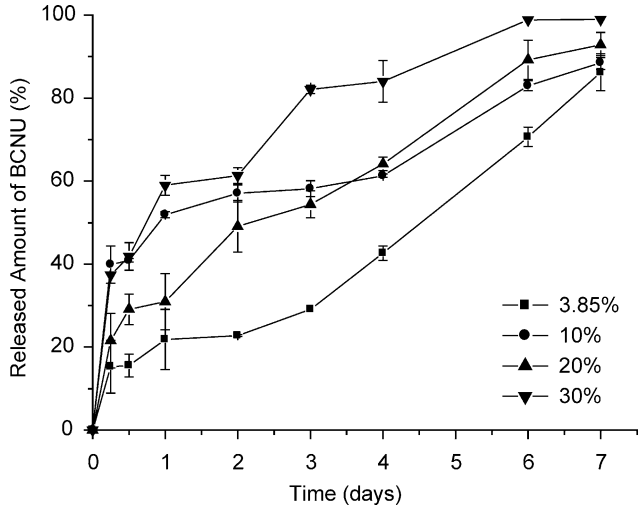

Fig. 1. Effect of the drug loading ratio on the release profiles of BCNU from PLGA wafers.

but also on the degradation of PLGA. Therefore, drug loading amount, molecular weight, and monomer ratio of copolymer are the major factors affecting the drug release rate and pattern. A period of sustained release estimated from approximately 12 h to 7 days while release amount increased continuously. This release pattern is mainly dependent on the diffusion of the drug through the polymer matrix that has many channels due to the polymer degradation after the water uptake.

3.2. In vitro antitumor activity

In the case of intact BCNU powder (Fig. 2), above 30 μg/ml BCNU showed *T*/*C* values more than 90% up to 3 days in comparison with control, but after 3 days, it did not reveal cytotoxicity to the cells. In the case of below 10 μg/ml BCNU powder, up to 3 days, it showed *T*/*C* values more than 60%, and after 3 days it also did not reveal cytotoxicity to the cells. In all the concentrations tested, BCNU powder did not reveal cytotoxicity to the 9L gliosarcoma cells after 3 days. From these results, we can suggest that the BCNU is continuously released from PLGA wafer as active form. In addition, these results strongly correlated with the results of in vitro release study.

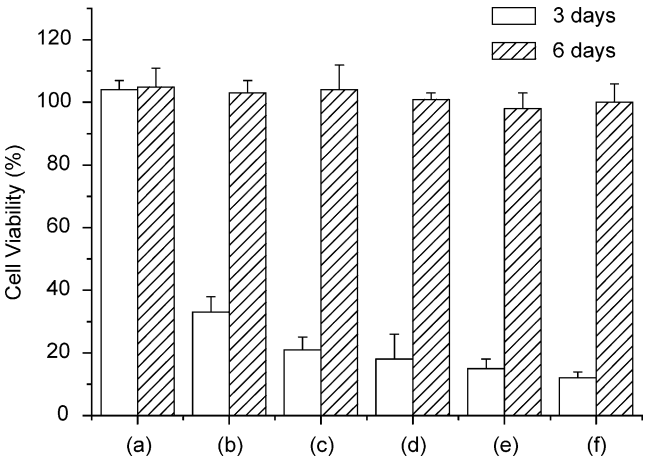

Fig. 2. Cytotoxicity of intact BCNU powder against 9L gliosarcoma cells in vitro. The BCNU powder were dissolved in cell culture medium at various concentrations (legend), and incubated for various time at 37 °C in the CO_2 incubator. Then, the solutions were added to the cells. Cell survival fractions were assessed after continuous exposure for 3 days by SRB assay. Legend: (a) control (non-treated), (b) 0.3, (c) 1, (d) 10, (e) 30, and (f) 100 μg/ml.

Cell morphology of 9L gliosarcoma treated BCNU-loaded PLGA wafers during 6 days in incubator was shown in Fig. 3. The wafers were plated on 6-well flat-bottomed plates on which the cells were attached, and their cell morphology on 3 and 6 days was compared with control. In case of control, the number of viable cells during 3 days was a little bit less than that obtained after 6 days. From the morphology of control, proliferation of cells was affected by incubation time. In case of 3.85% BCNU-loaded PLGA wafer, it affected the cell growth as the release amount of BCNU from the wafer containing 3.85% BCNU was enough to be effective for inhibition. As compared with 3 and 6 days, the cell density after 6 days was higher than that after 3 days. From this result, release amount of BCNU affects cell growth in 3.85% BCNU-loaded PLGA wafer. And, remaining viable cells could proliferate after 6 days. As the results of the cell morphology of 9L treated 10, 20, and 30% BCNU-loaded PLGA wafers, the cells densities after 6 days was more than that of initial period (not shown).

Cytotoxicity of BCNU-loaded PLGA wafers against 9L gliosarcoma cells in vitro is shown Fig. 4. This cell line was sensitive to the amount of released drug when it was exposed continuously to BCNU for 6 days.

In the case of PLGA wafer only (without BCNU, blank) in culture medium, all samples did not show cytotoxicity for the 9L gliosarcoma cells during test period. And the PLGA wafer also did not affect the growth of 9L gliosarcoma cells and the cells proliferate to 44.5×10^5 on the fourth day. Whereas, in all the groups including BCNU-loaded PLGA wafers, the above 60% of cells were dead for 3 days. And in case of treatment of 3.85% BCNU-loaded PLGA wafer in 9L gliosarcoma cells, the above 60% of the cells during 3 days and the above half of the cells were dead after 6 days. As the result of that, we supposed that BCNU was released from PLGA wafer continuously and the cytotoxicity against 9L gliosarcoma cells was affected significantly by BCNU without any effect of PLGA.

3.3. In vivo antitumor activity

Peritoneal application of BCNU is known to be less potent enough to affect the tumor growth than local administration with polymer [23] because of the short half-life of BCNU in vivo. From our preliminary cell culture study, we showed that BCNU was not active in a two-dimensional culture of tumor cells unless it was applied via a polymeric release system. There are two potential

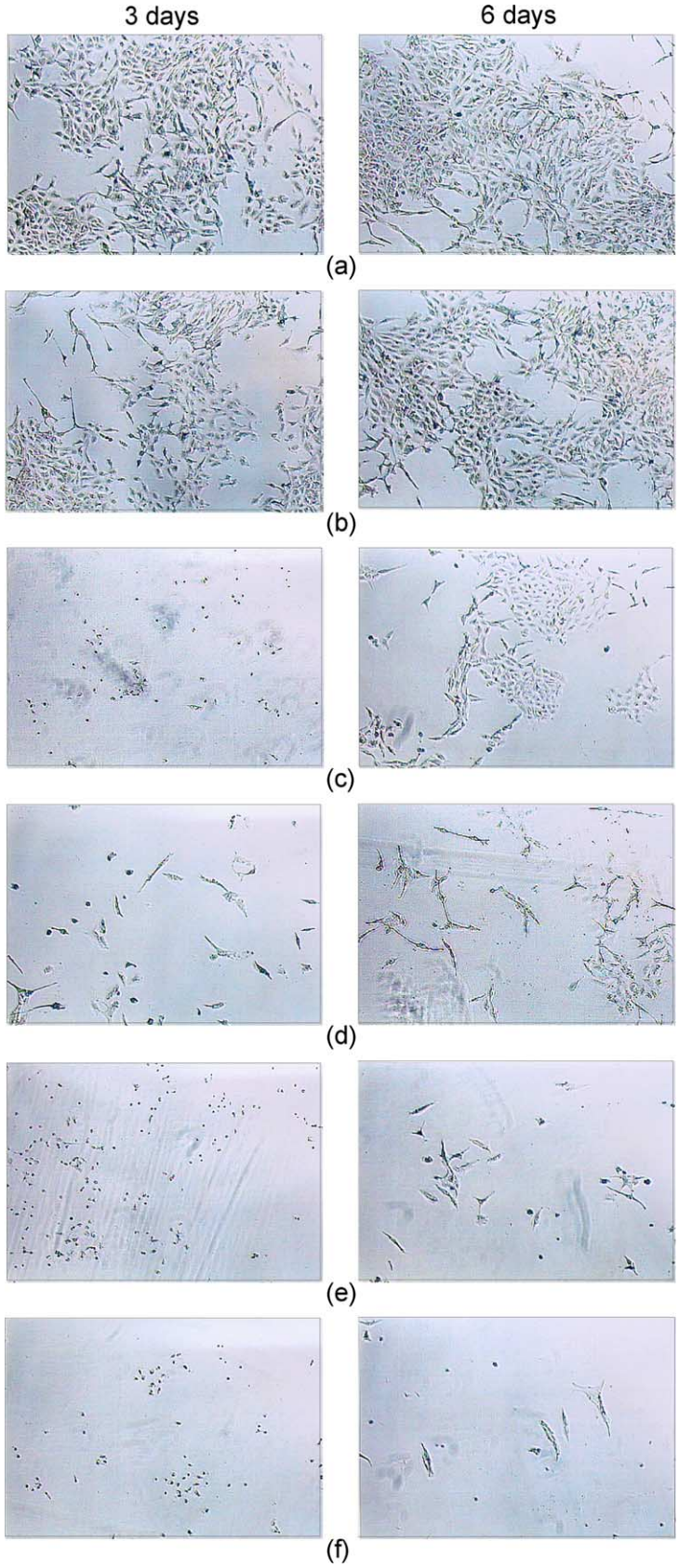

Fig. 3. Cell morphology of 9L gliosarcoma treated BCNU-loaded PLGA wafers during 6 days: (a) control (non-treated), (b) blank (PLGA only), (c) 3.85, (d) 10, (e) 20, and (f) 30% BCNU.

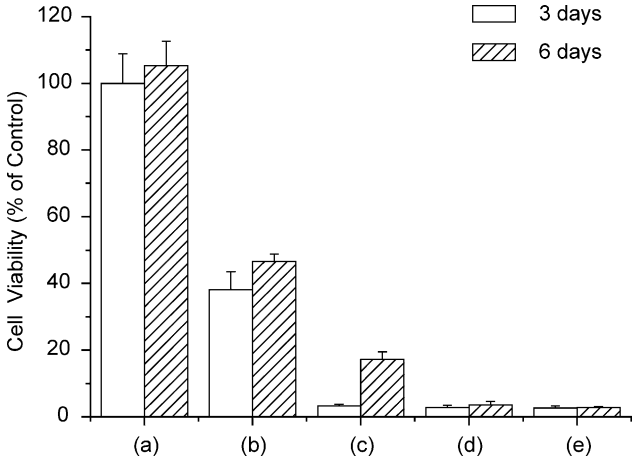

Fig. 4. Cytotoxicity of BCNU-loaded PLGA wafers against 9L gliosarcoma cells in vitro. The wafers were put in cell culture medium, and the solutions of the samples were transferred to the cells after the various incubation time at 37 °C in the CO_2 incubator. Cell survival fractions were assessed after continuous exposure for 3 days by SRB assay. (a) Blank (PLGA only), (b) 3.85%, (c) 10%, (d) 20%, and (e) 30% BCNU.

explanations for this phenomenon. The hydrophobic polymer might stabilize BCNU by creating an almost anhydrous environment that protects BCNU inside the matrix from degradation. Alternatively it could stabilize BCNU via acidification since pH drops to values around 4 inside pores of the polymer matrix during erosion, where BCNU is known to have a maximum stability. Either way the results illustrate that the activity of BCNU is the highest when applied locally via an implant that provides additional stabilizing mechanisms.

Five groups ($n=5$) were subjected to the experiment according to the BCNU-loading amount of 3.85, 10, 20, and 30% in the PLGA wafer in vivo antitumor activity test. Fig. 5 shows tumor volume changes of the treated group compared to the control (PLGA only) group. In control group the 9L gliosarcoma cells grew progressively reaching a tumor volume of $140{,}000 \times 3000\ mm^3$ in 45 days

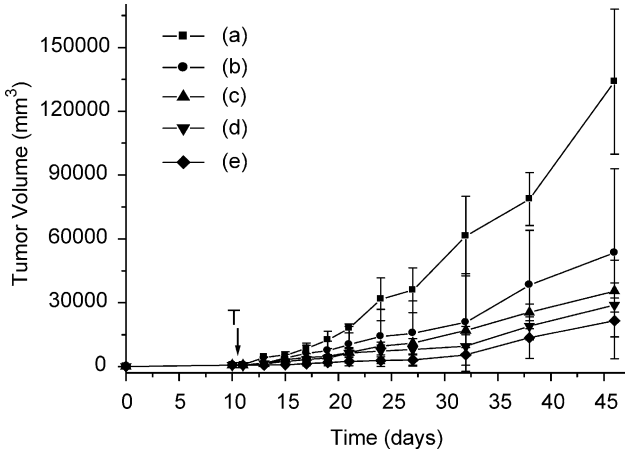

Fig. 5. Tumor volume changes of rats treated with BCNU-loaded PLGA wafers: (a) control (PLGA only), (b) 3.85, (c) 10, (d) 20, and (e) 30% BCNU; *T*: time at which treatment was initiated.

Table 1
Comparison of time to reach a tumor volume of 5000 mm^3 of experimental groups

Group no.	Drug (%)	The time in 5000 mm^3 (days) mean ± SD
Control	–	14.3 ± 0.3
1	3.85	15.9 ± 0.9
2	10	19.2 ± 0.7
3	20	19.9 ± 0.9
4	30	31.3 ± 0.3

Subcutaneous implantation of the BCNU-loaded polymer matrices at the tumor site resulted in tumor retardation and the tumor retardation was increased with increasing dose of BCNU in the wafer. This result suggested that the tumor was sensitive against local administration of BCNU at the tumor site and that the sensitivity persisted with time. The outcome of each treatment modality was evaluated by comparing the time required to reach a tumor volume of 5000 mm^3, a 10-fold increase in tumor volume after the treatment was initiated. In Table 1, the days for tumor to reach a volume of 5000 mm^3 of experimental groups were compared with the control group. The regional s.c. delivery of BCNU via the wafers containing different drug content resulted in a significant delay of 1.6–17.3 days ($P<0.001$) in the growth of the 9L gliosarcoma tumor compared with control (PLGA only) group.

4. Conclusion

Implantable BCNU-loaded PLGA wafers were developed by compressing molding of mixtures of BCNU and PLGA in simple and reproducible manner. The release rate of BCNU from PLGA wafer increased with the increase of BCNU loading amount and the release of BCNU was almost finished at 7 days. The wafers containing BCNU showed more effective cytotoxicity than intact BCNU powder due to its short half-life and BCNU released from BCNU-loaded PLGA wafer inhibited the proliferation of 9L gliosarcoma cells continuously. BCNU-loaded PLGA wafer delayed the growth of the tumors significantly and increasing the dose of BCNU in the wafer resulted in a more substantial retardation of the tumor. The study for the confirmation of these results using brain tumor model is in progress. In the future work, more attention will be paid to implants which can enhance the penetration depth of BCNU in the brain tissue for enhancing the therapeutic efficacy of recurrent gliomas.

Acknowledgements

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