

can be diagnosed as radiation necrosis, bevacizumab should be considered as a first-line treatment. We are currently trying to obtain the approval for the on-label use of bevacizumab for the treatment of radiation necrosis in Japan from the MHLW.

### Conflicts of Interest Disclosure

There is no conflict of interest to disclose for any of the authors.

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# Tetrakis(*p*-Carboranylthio-Tetrafluorophenyl)Chlorin (TPFC): Application for Photodynamic Therapy and Boron Neutron Capture Therapy

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Received 18 October 2014; revised 24 November 2014; accepted 2 December 2014

Published online 26 December 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24317

**ABSTRACT:** Carboranyl-containing chlorins have emerged as promising dual sensitizers for use in both photodynamic therapy (PDT) and boron neutron capture therapy (BNCT), by virtue of their known tumor affinity, low cytotoxicity in dark conditions, and their strong absorptions in the red region of the optical spectrum. Tetrakis(*p*-carboranylthio-tetrafluorophenyl)chlorin (TPFC) is a new synthetic carboranyl-containing chlorin of high boron content (24% by weight). To evaluate TPFC's applicability as sensitizer for both PDT and BNCT, we performed an *in vitro* and *in vivo* study using F98 rat glioma cells and F98 rat glioma-bearing brain tumor models. For the *in vivo* BNCT study, we used boronophenylalanine (BPA), which is currently used in clinical BNCT studies, via intravenous administration (i.v.) and/or used TPFC via convection-enhanced delivery (CED), a method for local drug infusion directly into the brain. In the *in vitro* PDT study, the cell surviving fraction following laser irradiation (9 J/cm<sup>2</sup>) was 0.035 whereas in the *in vitro* BNCT study, the cell surviving fraction following neutron irradiation (thermal neutron =  $1.73 \times 10^{12}$  n/cm<sup>2</sup>) was 0.04. In the *in vivo* BNCT study, the median survival time following concomitant administration of BPA (i.v.) and TPFC (CED) was 42 days (95% confidence interval; 37–43 days). © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:962–970, 2015

**Keywords:** absorption; blood–brain barrier; BNCT; boron-containing chlorin; CED; cell culture; CNS; osmotic pumps; PDT; TPFC

## INTRODUCTION

The prognosis of patients with malignant glioma, especially glioblastoma (GB), is poor. The median survival of GB patients is less than 2 years after the initial diagnosis,<sup>1</sup> with most recurrence occurring at the site of the original tumor. Therefore, more aggressive local therapies are necessary to eradicate unresectable tumor cells that invade adjacent normal brain tissue. Two adjuvant therapies with the potential to destroy these unresectable tumor cells are photodynamic therapy (PDT)<sup>2,3</sup> and boron neutron capture therapy (BNCT).<sup>4–7</sup> PDT and BNCT are two binary modalities for malignant glioma treatment that rely on the selective accumulation of a sensitizer within tumor tissue, followed by its activation upon irradiation with either red light (in PDT) or low-energy neutrons (in BNCT). The combination of PDT and BNCT using a single drug has several advantages, including increased therapeutic effect due to the targeting of different cellular components and/or mechanisms of tumor cell destruction.

Boronated porphyrins and their derivatives, in particular dihydroporphyrins or chlorins, are promising dual PDT and BNCT sensitizers because they usually have low dark toxicity, preferentially accumulate within tumor tissues and persist there for a considerable amount of time.<sup>8–10</sup> Moreover, their strong absorption of light wavelengths in the red region (~650 nm), which is characterized by the power of deeper penetration into most human tissues,<sup>11</sup> makes them ideal candidates for *in vivo* phototherapeutic applications;<sup>12</sup> on the other hand, their high boron content enhances the efficiency of their interaction with thermal neutrons. In 2008, a new carboranyl-containing chlorin, designated TPFC [tetrakis(*p*-carboranylthio-tetrafluorophenyl)chlorin (TPFC)], containing four boron clusters (24% boron by weight), was reported by our collaborator, Vicente and coworkers.<sup>13</sup> This report showed that TPFC was found to be non-toxic in the dark but showed extensive photosensitizing ability both *in vitro* and *in vivo* despite its relatively low singlet oxygen quantum yield. In particular, TPFC exhibited significant photosensitizing activity against highly pigmented melanotic melanoma tumors in mice.

In our study, we evaluated the potential of TPFC as a dual sensitizer for both PDT and BNCT using F98 rat glioma cells and F98 rat glioma-bearing brain tumor models. Although several carboranyl-containing chlorins and their derivatives have been reported as dual sensitizers for both PDT and BNCT,<sup>13–15</sup> in most of these studies, *in vivo* investigations were not conducted. Additionally, there were no reports of *in vivo* neutron irradiation study using a carboranyl-containing chlorin administered by convection-enhanced delivery (CED), a method for local drug infusion directly into the brain. This study is a first

**Abbreviations used:** BNCT, boron neutron capture therapy; BPA, boronophenylalanine; BSH, sodium borocaptate; CBE, compound biological effectiveness; CED, convection-enhanced delivery; CI, confidence interval; DMSO, dimethylsulfoxide; GB, glioblastoma; HpD, hematoporphyrin derivative; KUR, Kyoto University Research Reactor; LET, linear energy transfer; PBS, phosphate-buffered saline; PDD, photodynamic diagnosis; PDT, photodynamic therapy; RBE, relative biological effectiveness; TPFC, tetrakis(*p*-carboranylthio-tetrafluorophenyl)chlorin.

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Journal of Pharmaceutical Sciences, Vol. 104, 962–970 (2015)

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report of an *in vivo* neutron irradiation study using a new carboranyl-containing chlorin (TPFC) administered by CED.

## MATERIALS AND METHODS

### Boron Compounds

The TPFC was prepared as previously described.<sup>13</sup> TPFC was dissolved in dimethylsulfoxide (DMSO); the final DMSO concentrations never exceeded 1%. Boronophenylalanine (BPA) (L-isomer), which is currently used in clinical BNCT studies, was kindly supplied by the Stella Chemifa Corporation (Osaka, Japan) and was prepared as a fructose complex.<sup>16</sup>

### Cell Culture

F98 rat glioma cells produce infiltrating tumors in the brains of Fischer rats.<sup>17</sup> The tumors have been shown to be refractory to a number of treatment modalities, including radiation therapy.<sup>18</sup> Based on their *in vivo* histology, the F98 rat glioma cells have been characterized as anaplastic or undifferentiated glioma.<sup>19</sup> In the present study, F98 rat glioma cells were kindly obtained from Dr. Barth (Department of Pathology, Ohio State University, Columbus, Ohio). They were routinely cultivated in our laboratory in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and penicillin at 37°C in an atmosphere of 5% CO<sub>2</sub>. All the materials for the culture medium were purchased from Gibco Invitrogen (Grand Island, New York).

### *In Vitro* Cellular Uptake Study

In order to investigate the ability of TPFC for delivery of boron intracellularly, a cellular uptake was conducted, in which the cells were exposed to 10 µg <sup>10</sup>B/mL of TPFC or BPA for 2.5, 6, 12, and 18 h. First, F98 rat glioma cells were seeded in 100 mm dishes (BD Falcon™, Franklin Lakes, New Jersey), and the culture medium was exchanged for boron compounds-containing (TPFC, BPA) culture medium just before confluence. In this study, three 100 mm dishes for each designated time were used. After the completion of exposure, boron compounds-containing culture medium was removed, and then the cells were washed twice with 4°C phosphate-buffered saline (PBS) and detached with trypsin-ethylenediamine tetraacetic acid solution. Medium was then added, and the cells counted and sedimented (centrifugation: 214.2 G-force for 5 min). Cells were digested overnight with 1 N nitric acid solution, and boron uptake was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) using an iCAP6000 emission spectrometer (Hitachi High-Technologies, Tokyo, Japan). PBS and trypsin-ethylenediamine tetraacetic acid solution were purchased from Gibco Invitrogen, and the nitric acid solution was purchased from Wako Pure Chemical Industries (Osaka, Japan).

### *In Vitro* PDT Study

F98 rat glioma cells were incubated in culture media without TPFC and with two different doses of TPFC (2.5 and 5 µg <sup>10</sup>B/mL) for 18 h in 150 cm<sup>2</sup> flasks (TPFC<sub>eq</sub> \o\ac(0,R), Trasadingen, Switzerland). Following incubation, the cells were retrieved from the flasks, seeded onto 60 mm dishes (BD Falcon™) with 10<sup>4</sup> cells each and irradiated with visible light of 405 nm from a diode laser (Ball Semiconductor, Frisco, Texas). The cells were evenly irradiated at powers of 0, 4.5, 9, and

18 J/cm<sup>2</sup>. Following laser irradiation, the cells were seeded onto dishes, each with the same pre-determined number of cells, iteratively. All cells were cultured for 7 days, then fixed in methanol and stained with tripan blue. Colonies composed of more than 50 cells were counted and assessed by calculating the cell surviving fraction (= colony-forming assays).

### *In Vitro* BNCT Study

F98 rat glioma cells were transferred to the atomic reactor (KUR; Kyoto University Research Reactor) and incubated with the only culture medium (= control) and 10 µg <sup>10</sup>B/mL of TPFC or BPA-containing culture medium for 24 h. The cells were washed twice with PBS, detached with trypsin-ethylenediamine tetraacetic acid solution, and centrifuged at 1500 rpm for 5 min. The culture medium was then added to 1 mL of the cell suspension at a density of 1 × 10<sup>6</sup> cells/mL. Both types of cells were irradiated at KUR with reactor thermal neutron beams of 9.63 × 10<sup>8</sup> (n/cm<sup>2</sup>.s) for 0, 10, 20, 30 min at 1.0 MW reactor power. After neutron irradiation, colony-forming assays were performed as described above.

### Tumor Model

All male Fischer rats (200–250 mg, F344 NSlc; Japan SLC, Shizuoka, Japan) were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg) and placed in a stereotactic frame (Model 900; David Kopf Instruments, Tujunga, California). A midline scalp incision was made and the bregma was identified. A 1 mm burr hole was made in the right frontal region of the skull and a 22-gauge needle attached to a 25 µL syringe was inserted into the caudate nucleus using the same stereotactic coordinates, with the needle tip inserted 5 mm into the dura. An injection of 10<sup>6</sup> cells in 10 µL of serum free medium was administered at a rate of 1 µL/min. After the infusion, the needle was left in place for 3 min and the burr hole was then covered with bone wax and the scalp was sutured.

### Convection-Enhanced Delivery

Convection-enhanced delivery, a method for local drug infusion directly into the brain,<sup>20</sup> enables the distribution of any drugs homogeneously in the brain, keeping high concentration at the target site without mechanical damage to the surrounding normal tissue. CED depends on the bulk flow in the interstitial space produced by continuous slow infusion into the brain under low positive pressure. Using this method, it is theoretically possible to deliver drugs to regions where tumor cells invade microscopically with little accumulation in the blood and other organs. Moreover, because it does not depend on the molecular weight of the infused agent, this method allows the selection of many kinds of therapeutic agents and/or carriers, such as liposomes, dendrimers, and nanotubes.<sup>21–25</sup> Clinical trials of targeted toxins with epidermal growth factor receptor, transferin receptor, interleukin-13 receptor, and interleukin-4 receptor are reported in the literature.<sup>22,26–29</sup>

### *In Vivo* Biodistribution Study

For the biodistribution study, 10 days after tumor implantation, F98 rat glioma-bearing brain tumor models were generally anesthetized, placed in a stereotactic frame as described above, shaved and washed the skin over the rat's scalp and back. The midline scalp incision and a skull burr hole that were made when tumor models were made were re-opened and

5 mm-long cannula (ALZET Brain Infusion Kit 2; DURECT Corporation, Cupertino, California) were inserted into the burr hole through the skull. A mid-scapular incision was made, by spreading the subcutaneous tissue of the back to create a pocket for the infusion pump (ALZET Osmotic Pumps 2001D; DURECT Corporation), to be inserted. This infusion pump can administer compounds to animals over 24 h at a rate of 8  $\mu\text{L/h}$ . After the insertion of the infusion pump, a catheter leading to the brain cannula was attached to the subcutaneous pocket. Finally, the midline scalp incision and the mid-scapular incision were closed. The total amounts of TPFC administered via CED to each group of rats were 0.125, 0.25, and 0.5 mg per rat. We then assayed the  $^{10}\text{B}$  concentration of tumor, blood, normal brain, liver, spleen, kidney, heart, lung, skin, and muscle by ICP-AES. In this study, we sampled normal tissues from both hemispheres and defined the normal tissue from the tumor-bearing hemisphere as ipsilateral normal brain and the normal tissue from the other hemisphere as contralateral normal brain. The normal brain of the ipsilateral side, at least a couple of millimeters away from the tumor border, was sampled with approximately 1 g for  $^{10}\text{B}$  content measurement. Similarly, the normal brain of the contralateral side, symmetric relative to the ipsilateral sampling, was also sampled for the boron measurement. Normal brain for the tumor-to-normal brain ratio ( $=\text{T/N}$ ) determinations used normal brain from the contralateral side.

#### In Vivo BNCT Study

For the therapy study, neutron irradiation was performed in an atomic reactor (KUR) 14 days after the implantation of  $10^3$  F98 rat glioma cells. The radiation field delivered to the tissue during irradiation was composed mainly of thermal neutrons ( $<0.53$  eV), gamma photons, and heavy-charged particles ( $^4\text{He}$ ,  $^7\text{Li}$ ,  $^1\text{H}$ , and  $^{14}\text{C}$ ) from the  $^{10}\text{B}$  (n,  $\alpha$ ) $^7\text{Li}$  capture reaction and the  $^{14}\text{N}$ (n, p) $^{14}\text{C}$  reaction with normal tissue nitrogen. The fast neutron level ( $>10,000$  eV) was very low and therefore ignored. The thermal neutron flux was measured using 3 mm long gold wires placed on the surface of tumors. The average of the thermal neutron flux was  $6.08 \times 10^8$  (n/cm $^2$ /s) and the thermal neutron fluence became  $2.19 \times 10^{12}$  n/cm $^2$  for 60 min irradiation. The equivalent dose was calculated using the equations below.<sup>30</sup>

$$\text{Equivalent dose (Gy-Eq)} = D_B \times \text{CBE}_B + D_N \times \text{RBE}_N + D_\gamma \times \text{hours}$$

Gy-Eq refers to a biologically equivalent X-ray dose that can give radiation effects totally to the tissue,  $D_B$  (boron dose) =  $7.43 \times 10^{-14} \times$  boron concentration ( $\mu\text{g}^{10}\text{B/g}$ )  $\times \Phi$  thermal neutron fluence (Gy),  $D_N$  (nitrogen dose) =  $6.78 \times 10^{-14} \times$  nitrogen concentration (weight%)  $\times \Phi$  thermal neutron fluence (Gy),  $D_\gamma$  (gamma-ray dose) = 0.83 Gy/h,  $\text{RBE}_N$  is the RBE (relative biological effectiveness) for  $D_N$  and it equals 2.5,  $\text{CBE}_B$  is the CBE (compound biological effectiveness) for  $D_B$  and, in the case of BPA, this value is 3.8 for the tumor tissue and 1.35 for the normal brain.<sup>31–33</sup> The nitrogen concentration (%) is estimated as 4% and the RBE for  $D_\gamma$  is 1.0.

Fischer rats were transported to the KUR, randomized at 14 days after implantation, and divided into five experimental groups composed of 6–10 animals each. These groups included untreated control and irradiated control animals without boron compounds. The other three groups were composed of TPFC administered by CED, BPA administered by intravenous

injection, and a combination of TPFC and BPA group. Two and a half hours after intravenous administration of BPA (250 mg/kg b.w.) and/or twelve hours after termination of CED using TPFC (0.25 mg/rat), neutron irradiation was initiated. All irradiated rats were anesthetized and put in a whole body stabilizer with their heads placed out of the shield. The heads were irradiated at a reactor power of 1.0 MW for 1 h. After neutron irradiation, the neutron irradiated animals and the neutron unirradiated animals remained at KUR for observation. We checked the survival time of all rats.

#### In Vitro/In Vivo Fluorescence Microscopy

For the *in vitro* fluorescence microscopy study, F98 rat glioma cells were seeded in a two-well chamber mounted on glass slides with a cover (Thermo Fisher Scientific Inc., Rochester, NY), and the culture medium without TPFC was exchanged for TPFC-containing culture medium just before confluence. The cells were exposed to 20  $\mu\text{g}$   $^{10}\text{B}/\text{mL}$  of TPFC for 24 h. After exposure, the glass slides were washed with 4°C PBS and the two-well chamber was removed. The nuclei specific Hoechst dye (Hoechst 33342; Lonza) was added (10  $\mu\text{g}/\text{mL}$ ) and the glass slides mounted onto cover glasses using DPX Mountant for histology (Fluka Biochemika 44581, Germany). The two-well chamber slides were observed using an inverted fluorescence microscope system (BZ-8000; KEYENCE, Japan).

The *in vivo* fluorescence microscopy study was conducted twenty four hours after termination of CED administration of TPFC to F98 rat glioma-bearing brain tumor model. The rats were euthanized and strip preparations of the brains were made. Similar to the *in vitro* fluorescence microscopy study, the strip preparations of brain were observed using an inverted fluorescence microscope system (BZ-8000; KEYENCE).

#### Statistical Analysis

Values are presented as means  $\pm$  SD. Statistical analysis was performed by the Kaplan Meier method using JMP<sup>®</sup> Pro 10 (SAS Institute Inc., Cary, North Carolina).

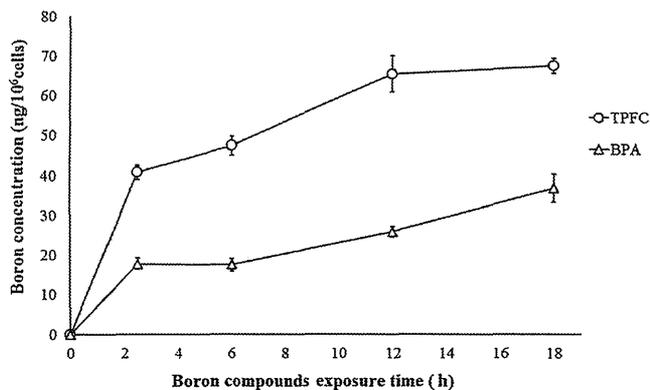
## RESULTS

#### In Vitro Cellular Uptake Study

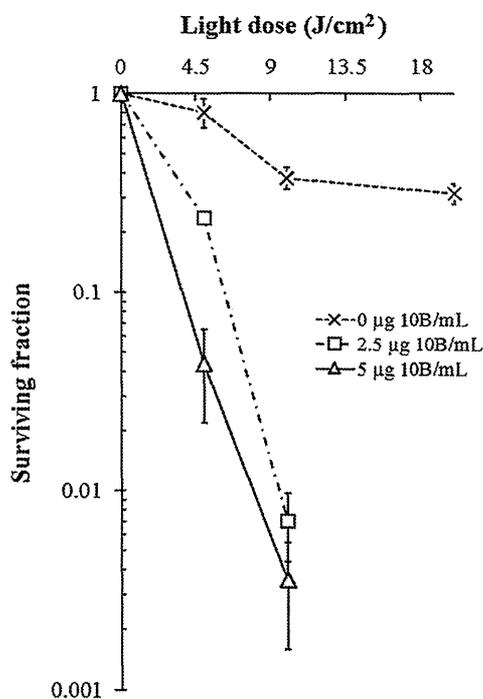
The ICP-AES measured cellular boron concentrations obtained in the *in vitro* cellular delivery study using TPFC were  $40.8 \pm 1.8$ ,  $47.4 \pm 2.4$ ,  $65.4 \pm 4.7$ , and  $67.4 \pm 1.9$  ng $^{10}\text{B}/10^6$  cells after 2.5, 6, 12, or 18 h of exposure, respectively. On the other hand, the values obtained using BPA under the same conditions were  $17.9 \pm 1.4$ ,  $17.6 \pm 1.6$ ,  $25.8 \pm 1.3$ , and  $36.7 \pm 3.5$  ng $^{10}\text{B}/10^6$  cells after 2.5, 6, 12, or 18 h of exposure, respectively. These results are shown in Figure 1.

#### In Vitro PDT Study

The cytotoxicity of TPFC determined by laser irradiation using a colony-forming assay showed the surviving fraction of cells following exposure to TPFC (0, 2.5, and 5  $\mu\text{g}$   $^{10}\text{B}/\text{mL}$  for 18 h) and laser irradiation to be:  $0.238 \pm 0.012$ ,  $0.007 \pm 0.003$ , and unmeasurable value (for TPFC = 2.5  $\mu\text{g}$   $^{10}\text{B}/\text{mL}$  for 18 h), and  $0.043 \pm 0.021$ ,  $0.004 \pm 0.002$ , and unmeasurable value (for TPFC = 5  $\mu\text{g}$   $^{10}\text{B}/\text{mL}$  for 18 h), using 4.5, 9, and 18 J/cm $^2$  light dose, respectively. The unmeasurable value means no colonies on dishes ( $<0.000$ ) owing to the high photocytotoxicity of TPFC under the experimental conditions. The surviving fractions for



**Figure 1.** TPFC's cellular uptake of boron ( $\text{ng}^{10}\text{B}/10^6$  cells) was about twice as high as BPA's cellular uptake of boron on each time.

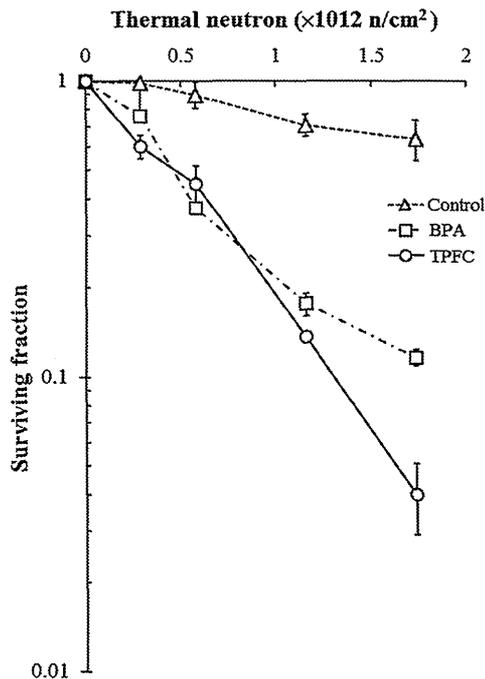


**Figure 2.** Colony-forming assay using F98 rat glioma cells exposed to 0 (control), 2.5, and 5  $\mu\text{g}^{10}\text{B}/\text{mL}$  of TPFC and irradiated with light doses of 0, 4.5, 9, and 18  $\text{J}/\text{cm}^2$ , respectively. The most efficient PDT-induced tumoricidal effect was achieved when the cells were irradiated with 9  $\text{J}/\text{cm}^2$ , 18 h after exposure to 5  $\mu\text{g}^{10}\text{B}/\text{mL}$  of TPFC (less than 0.05).

the laser-only control (without TPFC) were  $0.805 \pm 0.135$ ,  $0.377 \pm 0.046$ , and  $0.315 \pm 0.036$ , using 4.5, 9, and 18  $\text{J}/\text{cm}^2$  light dose, respectively. These results are shown in Figure 2.

#### In Vitro BNCT Study

The cytotoxicity of TPFC upon neutron irradiation was also evaluated with a colony forming assay, performed on F98 rat glioma cells 7 days after irradiation with the reactor thermal neutron beam. The results are shown in Figure 3. For the control groups exposed to the neutron beam without boron



**Figure 3.** F98 rat glioma cells were exposed to culture medium without boron compound (= control), with 10  $\mu\text{g}^{10}\text{B}/\text{mL}$  of BPA or TPFC for 24 h. Then they were irradiated with thermal neutron, respectively. BPA's cell surviving fraction following thermal neutron for 30 min was 0.12. On the other hand, TPFC's cell surviving fraction following thermal neutron for 30 min was 0.04.

compounds (BPA or TPFC), the surviving fraction decreased only slightly, with increasing thermal neutron fluence, with determined values of 1, 0.98, 0.90, 0.71, and 0.64 for each neutron fluence of 0, 0.289, 0.578, 1.156, and  $1.733 \times 10^{12} \text{ n/cm}^2$ , respectively. On the other hand, the survival ratios of tumor cells treated with BPA or TPFC decreased exponentially with the increase of neutron fluence. The cytotoxicity of BNCT was especially significant in the TPFC group, with the determined cell surviving fraction following thermal neutron for 30 minutes of 0.04.

#### In Vivo Biodistribution Study

The boron concentrations following CED of TPFC to the F98 rat glioma-bearing brain tumor models at 24 h after termination of CED were also observed at different TPFC doses (0.125, 0.25, and 0.5 mg TPFC/rat). The tumor boron concentrations showed the highest concentrations at a dose of 0.25 mg TPFC/rat; the boron concentrations at a dose of 0.125, 0.25, and 0.5 mg TPFC/rat were  $22.69 \pm 1.79$ ,  $40.35 \pm 11.78$ , and  $29.56 \pm 10.44 \mu\text{g}^{10}\text{B}/\text{g}$ , respectively. Low concentrations were observed in the ipsilateral brain, the contralateral brain, and the blood at the different TPFC doses:  $0.21 \pm 0.16$ ,  $2.01 \pm 1.30$ , and  $0.23 \pm 0.35$  in the ipsilateral brain,  $0.05 \pm 0.03$ ,  $0.97 \pm 0.40$ , and  $0.14 \pm 0.23$  in the contralateral brain, and  $0.82 \pm 0.61$ ,  $0.46 \pm 0.19$ , and  $0.26 \pm 0.01$  in blood, respectively. Tumor/normal brain ratio and tumor/blood ratio of boron at the different TPFC doses were 454, 42, and 211 for the tumor/normal brain ratio and 28, 88, and 114 for the tumor/blood ratio, respectively.

**Table 1.** Boron Concentrations in Brain Tumors in Rats Bearing F98 Gliomas to which TPFC was Administered by Convection-Enhanced Delivery

TPFC Concentration	Time (h) <sup>a</sup>	Boron concentration (μg/mL)				Ratio	
		Tumor	Ipsilateral Brain	Contralateral Brain	Blood	T/N <sup>b</sup>	T/B <sup>c</sup>
0.125 mg TPFC/rat (N = 11)	0 (N = 3)	13.02 ± 0.78	0.09 ± 0.02	0.07 ± 0.01	1.13 ± 0.20	186	12
	24 (N = 5)	22.69 ± 1.79	0.21 ± 0.16	0.05 ± 0.03	0.82 ± 0.61	454	28
	48 (N = 3)	9.75 ± 3.96	0.05 ± 0.01	0.01 ± 0.01	0.33 ± 0.04	975	30
0.25 mg TPFC/rat (N = 3)	24	40.35 ± 11.78	2.01 ± 1.30	0.97 ± 0.40	0.46 ± 0.19	42	88
0.5 mg TPFC/rat (N = 3)	24	29.56 ± 10.44	0.23 ± 0.35	0.14 ± 0.23	0.26 ± 0.01	211	114

<sup>a</sup>Hours after termination of convection-enhanced delivery of TPFC.<sup>b</sup>T/N is tumor/normal brain (ipsilateral brain).<sup>c</sup>T/B is tumor/blood.**Table 2.** Boron Concentrations in Organs Other than Brains in Rats Bearing F98 Gliomas to which TPFC was Administered by Convection-Enhanced Delivery

TPFC Concentration	Time (h) <sup>a</sup>	Boron Concentration (μg/mL)						
		Liver	Spleen	Kidney	Heart	Lung	Skin	Muscle
0.125 mg TPFC/rat (N = 8)	0 (N = 3)	0.20 ± 0.11	0.52 ± 0.13	0.16 ± 0.08	0.11 ± 0.05	0.19 ± 0.12	0.04 ± 0.06	0.01 ± 0.02
	24 (N = 5)	0.19 ± 0.03	0.61 ± 0.42	0.24 ± 0.04	0.21 ± 0.01	0.40 ± 0.11	0.08 ± 0.06	0.03 ± 0.03

<sup>a</sup>Hours after termination of convection-enhanced delivery of TPFC.

Table 1 summarizes the biodistribution data (tumor, ipsilateral brain, contralateral brain, and blood) for TPFC administered by CED. We first focused our efforts on the differences corresponding to the time after termination of CED of TPFC up to 48 h. The boron concentrations in F98 tumor nodules were 13.02 ± 0.78, 22.69 ± 1.79, and 9.75 ± 3.96 μg <sup>10</sup>B/g at 0, 24, and 48 h after termination of CED of 0.125 mg/rat TPFC, respectively. The boron concentrations in the ipsilateral brain, the contralateral brain, and the blood also increased up to 24 h after termination of CED and decreased at 48 h after termination of CED. The corresponding tumor/normal brain ratios for the three different times were 186, 454, and 975, respectively.

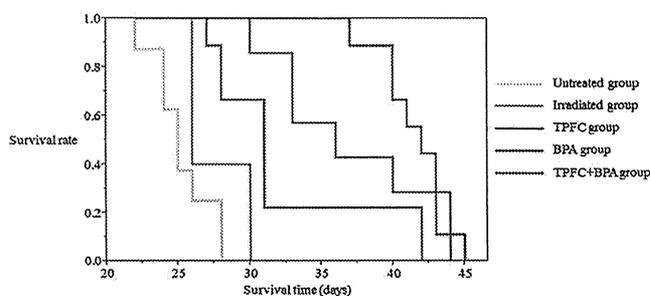
Table 2 summarizes the biodistribution data (liver, spleen, kidney, heart, lung, skin, and muscle) obtained for TPFC administered by CED. The boron concentrations of all organs showed very low dose.

### In Vivo BNCT Study

Median survival times of control groups (untreated and irradiated groups), a TPFC administered by CED group, a BPA administered by intravenous injection group and a combination of TPFC and BPA group were 25 days [95% confidence interval (CI); 22–28 days], 26 days (95% CI; 26–33 days), 31 days (95% CI; 27–42 days), 36 days (95% CI; 30–44 days), and 42 days (95% CI; 37–43 days), respectively. There is no over-lap in 95% CI between TPFC group and untreated group/irradiated group. There is over-lap in 95% CI between a TPFC group and a BPA group and a combination group. These results are shown in Figure 4.

### In Vitro/In Vivo Fluorescence Microscopy.

*In vitro* fluorescence microscopy showed the intracellular chlorin fluorescence (Fig. 5a) and the images observed from the co-localization experiment using the nucleus-specific Hoechst dye (Fig. 5b). These results show that TPFC was taken up into the cells and also localized in the nuclei (Fig. 5c). *In vivo*

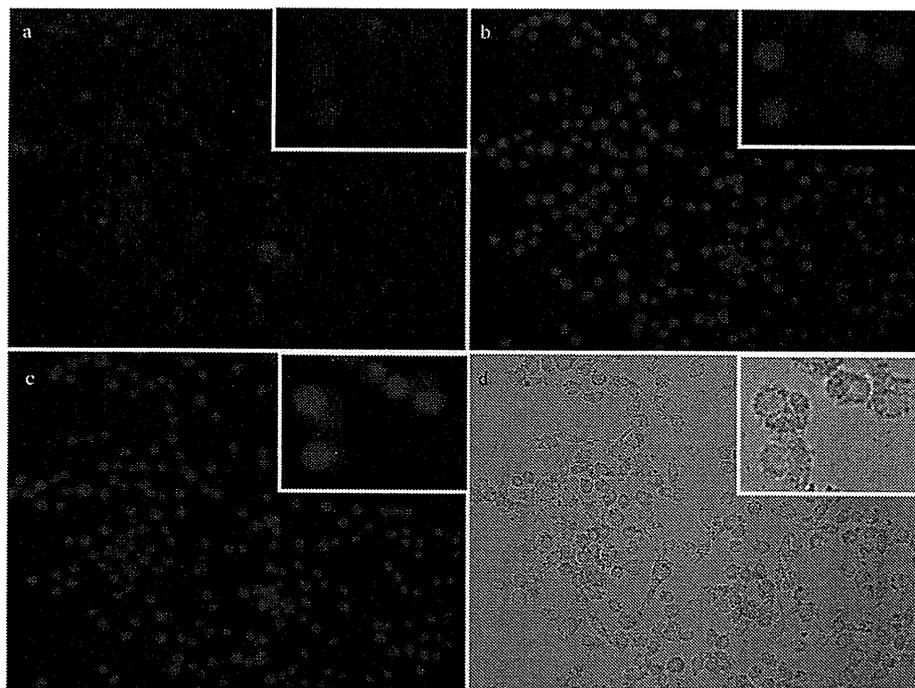


**Figure 4.** Median survival times of untreated group, irradiated group, TPFC group, BPA group, and TPFC + BPA group were 25 days (95% CI; 22–28 days), 26 days (95% CI; 26–30 days), 31 days (95% CI; 27–42 days), 36 days (95% CI; 30–44 days), and 42 days (95% CI; 37–43 days), respectively (yellow, green, blue, brown, and red line indicated untreated, irradiated, TPFC, BPA, and TPFC and BPA group, respectively). It was significantly between TPFC group and TPFC + BPA group ( $p$  value = 0.007 by Log-rank test and 0.004 by Wilcoxon test). However, it was no significantly between BPA group and TPFC + BPA group ( $p$  value = 0.350 by Log-rank test and 0.133 by Wilcoxon test). And it was no significantly between TPFC group and BPA group ( $p$  value = 0.110 by Log-rank test and 0.063 by Wilcoxon test).

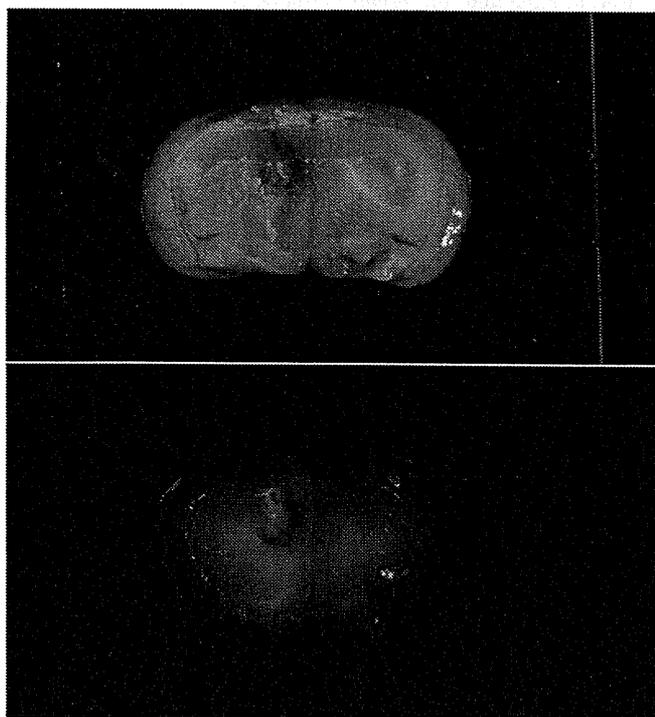
fluorescence microscopy also showed fluorescence of tumor, as shown in Figure 6.

## DISCUSSION

PDT is a localized therapy that relies on the specific uptake of a photosensitizer in the tumor relative to the surrounding normal tissue, followed by laser irradiation for activation of the photosensitizer.<sup>12</sup> The photoactivation of the sensitizer causes oxidative damage to a variety of cellular targets via the release of singlet oxygen and other reactive oxygen species, with subsequent tumor necrosis. To date, the clinical trials with PDT employed as an adjuvant treatment for human gliomas have used



**Figure 5.** (a) Fluorescence of chlorin (TPFC), (b) nuclear fluorescence by the Hoechst dye (excitation wavelength was 340–380 nm), (c) merged image (magnification of all images:  $\times 400$ ), and (d) bright field image.



**Figure 6.** *In vivo* fluorescence microscopy also showed fluorescence of tumor. Red fluorescence indicated the tumor and green fluorescence indicated the normal brain.

the poorly defined heterogeneous porphyrin mixture hematoporphyrin derivative (HpD) or its more enriched commercial preparation, Photofrin,<sup>2</sup> which does not contain boron. These compounds have several draw backs. The most significant are cutaneous photosensitivity lasting up to 8 weeks following treatment,<sup>34–36</sup> they are a complex mixture of porphyrins which makes accurate study difficult,<sup>37</sup> and the wavelength of light absorption is not optimal for tissue penetration.<sup>38,39</sup> Chlorin photosensitizers show great promise for use in PDT.<sup>40–45</sup> They have enhanced molar extinction and electronic absorption peaks at longer wavelengths than HpD, allowing for both increased tissue penetration and enhanced tumor photodestruction.<sup>40–42</sup> Furthermore, chlorins do not appear to cause severe cutaneous photosensitization after 24 h.<sup>41–43</sup> In 2008, a new carboranyl-containing chlorin (TPFC) was reported.<sup>13</sup> As TPFC is a boron-containing chlorin of high boron content (24% by weight), it is a promising dual sensitizer for the PDT and BNCT of gliomas.

In a similar fashion to PDT, BNCT is a targeted chemoradiation therapy that significantly increases the therapeutic ratio relative to conventional radiotherapeutic modalities. In BNCT, a  $^{10}\text{B}$ -labeled compound delivers therapeutic concentrations of  $^{10}\text{B}$  ( $\sim 30 \mu\text{g } ^{10}\text{B/g}$  tumor) to the target tumor, with high tumor-to-blood and tumor-to-normal-tissue ratios and low cytotoxicity.<sup>4,5</sup> Subsequently the tumor is irradiated with epithermal neutrons that become thermalized at a certain depth within the tissues. The short range ( $< 10 \mu\text{m}$ ) of the  $\alpha$  and  $^7\text{Li}$  high linear energy transfer (high-LET) particles released from the  $^{10}\text{B}(n, \alpha)^7\text{Li}$  neutron capture reaction makes the tumor microdistribution of  $^{10}\text{B}$  critically important in BNCT. As the high-LET particles are highly cytotoxic, their killing effect

depends on the site of generation. These characteristics contribute to the tumor selectivity and strong tumoricidal activity of BNCT, with negligible damage to normal tissue. Therefore, if sufficient quantities of boron can be selectively delivered to tumor tissues, BNCT could be an ideal tumor-selective particle beam irradiation local therapy for malignant gliomas. Clinically, BPA and/or sodium borocaptate (BSH) are currently available for BNCT as boron delivery agents. BPA is a boronated derivative of an essential amino acid (L-phenylalanine) that is actively taken up by tumor cells, presumably via the amino acid transport mechanism. BSH, on the other hand, is believed to preferentially accumulate within tumor tissue via a partially destroyed or leaky blood brain barrier. We have used both of these boron delivery agents in combination in clinical BNCT studies, and have previously reported on the survival benefit from BNCT for newly diagnosed GB patients<sup>6</sup> as well as for recurrent malignant glioma patients.<sup>7</sup> However, the present results using BPA and/or BSH are far from satisfactory, and the use of more effective boron delivery agents should provide enhanced clinical outcomes for BNCT. The so-called third-generation of boron delivery agents,<sup>5</sup> including boronated porphyrin derivatives, carboranyl-containing chlorins, molecular-targeted agents (e.g., to EGFR), and liposome-linked boron delivery agents could potentially greatly increase the efficacy of BNCT. Among these boron delivery agents, carboranyl-containing chlorin TPFC is particularly promising because of its high boron content compared with other reported boron-containing chlorins, and also because TPFC has shown high efficacy as photosensitizer for PDT.<sup>13</sup>

In this study, our results show the efficacy of TPFC in both the *in vitro* PDT study (Fig. 2) and in the *in vitro* BNCT study (Fig. 3), TPFC was highly phototoxic and showed equal or superior performance compared with BPA upon neutron irradiation (Fig. 3). However *in vivo* TPFC did not show as high antitumor effect than BPA following neutron irradiation (Fig. 4). Although there are no great differences between the TPFC, BPA, and the combination of TPFC and BPA groups, the later combination of TPFC and BPA group showed the tendency to extend the survival time. It is possible that if PDT was followed by BNCT, greater survival times could have been observed. In the fluorescence microscopy experiment, TPFC was shown to be taken up by F98 rat glioma cells *in vitro* and by F98 rat glioma tumor *in vivo* (Figs. 5 and 6). However, the sites of localization of TPFC might be different following different routes of administration. Our results suggest that TPFC has promise as dual sensitizer for both PDT and BNCT of gliomas. Additionally, TPFC could also be used intraoperatively for photodynamic diagnosis (PDD) and fluorescence-guided resection of brain tumors.

Pre-operative administration of a carboranyl-containing chlorin has a number of advantages in the clinical setting. As shown in Figure 6, carboranyl-containing chlorins could be useful in PDD and in fluorescence-guided resection of brain tumors during surgery. Using fluorescence-guided resection of such tumors during surgery, the resection rate can be augmented, with expected further improvements in patient prognosis.<sup>46</sup> In addition, carboranyl-containing chlorins can be used with intraoperative PDT and post-operative BNCT. Although the initial results with commonly used photosensitizers for PDT such as Photofrin (or its unpurified form HpD) were encouraging, treatment failures did occur, mainly due to the limited penetration of light of 630 nm wavelength (=Photofrin and HpD) into the brain. Penetration of light of 630 nm, recorded by Muller and

Wilson,<sup>47,48</sup> for tumor, brain infiltrated by tumor and normal brain is  $2.9 \pm 1.5$ ,  $2.4 \pm 1.2$ , and  $1.5 \pm 0.4$  mm, respectively. However, TPFC has a large molar coefficient at 652 nm ( $\epsilon \sim 30,000$  in DMSO),<sup>13</sup> which is characterized by the power of deep penetration into most human tissues. Nevertheless PDT alone will probably not be sufficient for malignant glioma treatment. In cases with deep lesions, PDT alone may be inadequate to achieve complete tumor treatment, and it would be preferable in such cases to use BNCT as a supplementary treatment, using a boron-containing photosensitizer such as TPFC. Fairchild et al.<sup>49</sup> reported that thermal and epithermal neutrons are transported to a depth of approximately 10 cm, and BNCT has been shown to be adequate for the treatment of deep lesions. As boron-containing chlorins can be effective for BNCT as boron delivery agents while retaining their photosensitizer ability, the limited penetration of light can be overcome using a combination of BNCT and PDT for the treatment of human gliomas.

## CONCLUSIONS

The present study demonstrates that TPFC is a promising dual sensitizer for PDT and BNCT of F98 rat gliomas. In *in vitro* studies, TPFC is shown to be extremely phototoxic and to localize near the cell nuclei. In addition, both *in vitro* and *in vivo*, TPFC was efficient at treating F98 rat gliomas, comparable to known BPA. Interestingly, an *in vivo* study using a combination of TPFC and BPA showed the greatest rat survival times (mean value of 42 days), compared to TPFC alone (mean of 31 days) and BPA alone (mean of 36 days). Additionally, TPFC could be used in PDD and fluorescence-guided resection, as it is highly fluorescent.

## ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research (C) (20340549) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT) to S.K. and by the United States National Institutes of Health, grant number R01 CA 098902 to M.G.H.V. We thank Dr. Barth (Department of Pathology, the Ohio State University) for providing the F98 rat glioma cells.

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*Effect of boron neutron capture therapy  
for recurrent anaplastic meningioma: an  
autopsy case report*

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**Brain Tumor Pathology**

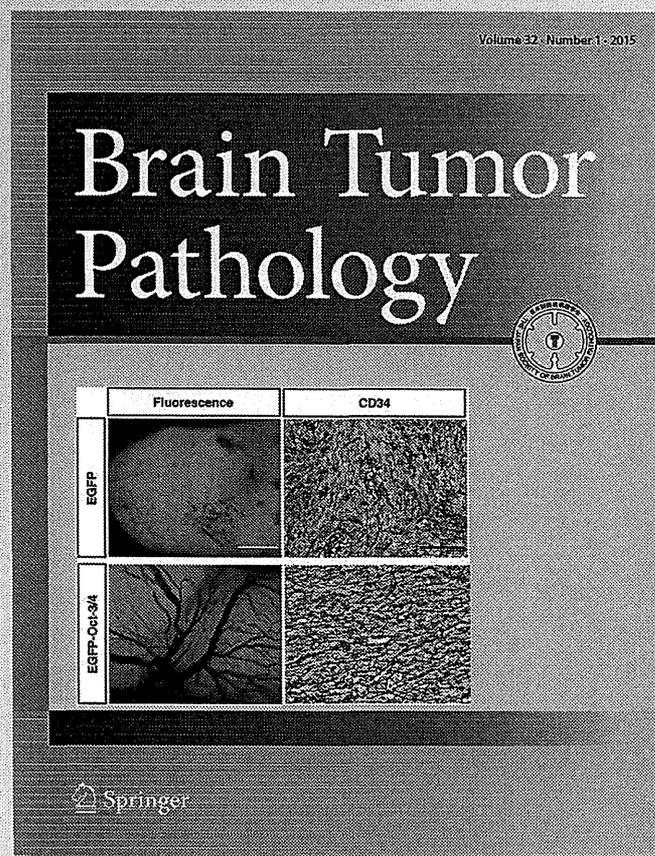
ISSN 1433-7398

Volume 32

Number 1

Brain Tumor Pathol (2015) 32:61-65

DOI 10.1007/s10014-014-0189-x



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## Effect of boron neutron capture therapy for recurrent anaplastic meningioma: an autopsy case report

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Received: 27 February 2014 / Accepted: 14 April 2014 / Published online: 8 May 2014  
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**Abstract** A 70-year-old woman died of systemic metastasis from anaplastic meningioma and underwent autopsy. The patient underwent twice total removal of the right sphenoid ridge meningioma 2 years ago. The tumor recurred 3 times, and then stereotactic radiotherapy was employed. Boron neutron capture therapy (BNCT) was performed for the fourth local recurrence and an additional new lesion. Proliferative activity of the newly developed meningioma, which had been treated with BNCT only, was significantly lower than that of untreated metastatic liver tumor, as well as that of the meningioma specimen obtained at the second surgery. Our pathological findings demonstrated, for the first time, the therapeutic effect of BNCT on anaplastic meningioma at an early stage (2.5 months).

**Keywords** Anaplastic meningioma · Autopsy · Boron neutron capture therapy · Bevacizumab · Radiation necrosis

### Introduction

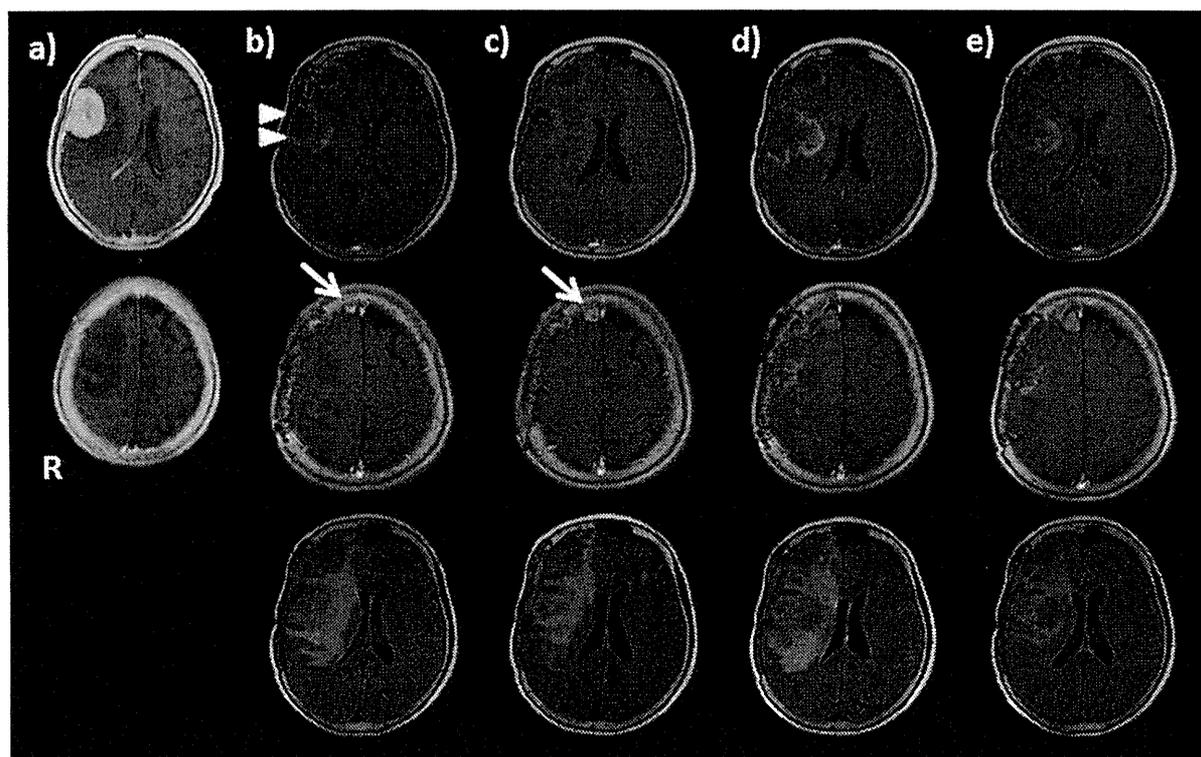
Meningioma accounts for 30 % of brain tumors, more than 80 % of which are benign tumor (WHO grade 1). Anaplastic meningioma is classified in the WHO grade 3 tumor and accounts for only 1–2 % of meningiomas [1]. Anaplastic meningioma is pathologically diagnosed when the tumor cells have anaplastic features and more than 20 mitotic figures in 10 high power fields of 0.16 mm<sup>2</sup> [1]. The recurrence ratio of anaplastic meningioma is 50–80 % [1] with a 5-year survival ratio of 50–60 % [2]. Although the extracranial metastasis ratio of the whole meningioma is <1 %, that of anaplastic meningioma is reported as high as 43 % [3]. The most frequent target organ of metastasis is lung, followed by liver, lymph nodes, and bone [4]. There is no effective chemotherapy for primary and metastatic anaplastic meningioma. The current standard therapy for anaplastic meningioma is tumor resection followed by adjuvant radiation therapy. Additional radiation therapy increases the survival ratio and reduces the recurrent ratio from 80 to 57 % [2]. However, for recurred tumors, repeated resection is the only effective therapy and repeated radiation therapy is regarded as useless [2].

Boron neutron capture therapy (BNCT) is a targeted radiation approach that significantly increases the therapeutic ratio when compared with that of conventional radiotherapeutic modalities. A <sup>10</sup>B-labeled compound delivers higher concentrations of <sup>10</sup>B to the target tumor, relative to the surrounding normal tissues. This is followed by irradiation with thermal or epithermal neutrons that become thermalized at a certain depth within the tissues. Short range (about 9 μm) of the α and lithium-7 particles released from the <sup>10</sup>B (neutron, α) lithium-7 neutron capture reaction makes the microdistribution of <sup>10</sup>B critically important in therapy. Release of these particles constitutes

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**Fig. 1** Time course of the brain MR images (*upper and middle rows*: T1-weighted images with contrast enhancement, *lower row*: fluid attenuated inversion recovery (FLAIR) images). **a** The initial images. **b** At 18 months after the second surgery, enhancement of the lesion 1 (*arrow heads*) enlarged with noticeable surrounding edema. An additional recurrent tumor was found in the frontal region just anterior to the lesion 1 (lesion 2: *arrow*). **c** At 2 months after the

administration of bevacizumab (20 months after the second surgery), enhancement of the lesion 1 and surrounding edema remarkably reduced. **d** At 22 months after the second surgery, enhancement of the both lesions and surrounding edema increased again. **e** At 1 month after the BNCT (24 months after the second surgery), the both enhanced areas and surrounding edema reduced

high-linear energy transfer particle radiation. These characteristics of BNCT contribute to tumor selective and strong tumoricidal activity with minimal damage to the normal tissue [5].

In the present report, we present a case of recurrent anaplastic meningioma treated with BNCT. Although intracranial tumors were successfully controlled, the patient died of systemic metastasis 2.5 months after BNCT. Postmortem examination revealed that proliferative activity of the tumors treated with BNCT was remarkably reduced. The efficacy of BNCT at an early stage after treatment is histologically verified.

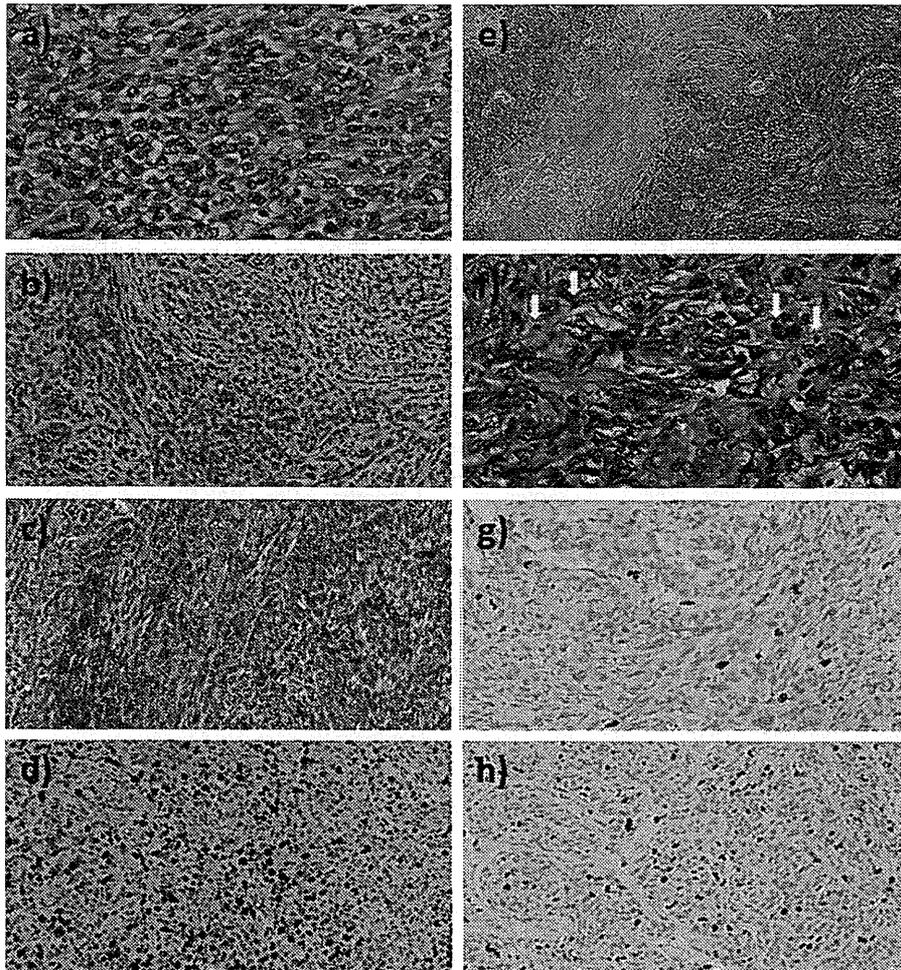
## Case report

### Clinical summary

A 67 year-old woman with recurrent right sphenoid ridge meningioma underwent total tumor removal

along with a part of the surrounding dura matter (Simpson grade 1). The tumor had been removed in another hospital 5 months previously (Fig. 1a). The pathological diagnosis was atypical meningioma (WHO grade 2). Although the pathological diagnosis of the recurrent tumor turned out more malignant, anaplastic meningioma WHO grade 3 (shown later), clinical course was carefully observed with repeated magnetic resonance (MR) imaging. Local recurrence invading the surface of the right cerebral cortex was observed (lesion 1) 5 months after the second surgery, which was treated with stereotactic radiotherapy (cyber knife). Since then, twice additional stereotactic radiotherapies were employed for the recurrent tumors (11 and 16 months after the second surgery, respectively).

Soon after the third stereotactic radiotherapy, namely at 18 months after the second surgery, enhancement of the lesion 1, which had recurred three times and been irradiated twice, enlarged with



**Fig. 2** Histological examination of the specimen obtained at the second surgery (*left*) and autopsy (*right*). The *arrows* indicate small pyknotic nuclei (*f*). Hematoxylin and Eosin staining (*a, e, f*), immunohistochemical staining for glial fibrillary acidic protein (*b,*

*negative*), EMA (*c, focally positive*), Ki67 (*d, g, h*),  $\times 5$  (*e*),  $\times 20$  (*b, c, d, g, h*),  $\times 40$  (*a, f*). MIB-1 LIs were 49.3 % in the meningioma before radiation therapy *d*, 3.6 % in the meningioma at autopsy (*g*), and 24.5 % in the untreated liver tumor at autopsy (*h*)

noticeable surrounding edema (Fig. 1b, arrow heads). Another recurrent tumor was found in the frontal region just anterior to the lesion 1 (lesion 2; Fig. 1b, arrow). When considering to adopt BNCT as the next treatment option, positron emission tomography (PET) using fluoride-18-labeled boronophenylalanine ( $^{18}\text{F}$ ]BPA) was performed. The lesion/normal (L/N) ratio was 2.0 in the lesion 1 and 3.3 in the lesion 2. Because the L/N ratio of the lesion 1 suggested radiation necrosis as a cause of brain edema, administration of the antivascular endothelial growth factor antibody (bevacizumab) was started. Immediately after this treatment, the enhancement and surrounding edema of the lesion 1 decreased (Fig. 1c), which, however, only lasted for 2 months (Fig. 1d).

The second  $^{18}\text{F}$ ]BPA-PET study demonstrated that the L/N ratio increased to 3.8 in the lesion 1 and 6.3 in the lesion 2, which indicated tumor progression rather than necrosis. Both lesions were treated with BNCT (Kyoto University Research Reactor Institute). Just after BNCT, bevacizumab was administered as schedule for the prevention of further radiation necrosis. At 1 month after BNCT, the enhancement and surrounding edema of the lesions slightly decreased (Fig. 1e). Simultaneously, bone metastases of the cervical spines, sternum and right femur were found and treated with conventional radiotherapy. At 2 months after the BNCT (26 months after the second surgery), multiple hepatic metastases with pleural and peritoneal dissemination were found, and then the patient died 1 month later. Autopsy was performed.

### Histopathological findings

Histological examination of the specimen obtained at the second surgery is shown in Fig. 2 (left column). The tumor cells of uneven size and high atypical nucleus were disorderly arranged and 31 mitotic figures in 10 high power fields of 0.16 mm<sup>2</sup> were observed. Immunohistochemical examination of the tumor cells revealed that the tumor cells were positive for vimentin and focally positive for epithelial membrane antigen (EMA) and negative for glial fibrillary acidic protein (GFAP), AE1/AE3, CD34, and progesterone receptor. The MIB-1 labeling index (LI) was as high as 49.3 %. The tumor was diagnosed as anaplastic meningioma WHO grade 3.

Histological examination of the autopsy specimen is shown in Fig. 2 (right column). The lesion 2 meningioma that was treated only with BNCT is shown in Fig. 2e, f. Although the patternless cell arrangement with irregular size nuclei is similar to the findings of the specimen obtained at the second surgery, focal tumor necrosis and small pyknotic nuclei (Fig. 2f, arrows) are also observed. No obvious difference in vascular density, evaluated with immunohistochemical staining of tumor vessels with CD-34 antibody, was observed between both meningioma tissue samples (before and after bevacizumab therapy) (data not shown). Mitotic figures are very few. The MIB-1 LI of the lesion 2 meningioma is 3.6 % (Fig. 2g), while that of the untreated metastatic liver tumor is 24.5 % (Fig. 2h).

### Discussion

There are only few reports of BNCT for malignant meningiomas [5, 6]. Kawabata et al. employed BCNT for 20 recurrent malignant meningioma patients who had been already treated with repeated resection and radiotherapy and achieved 64.5 % tumor volume reduction in the first 2 months after BNCT. Only 3 of 20 patients died due to local tumor progression. However, the survival length after BNCT was 13–44 months. Systemic metastasis was found in 6 cases, intracranial recurrence outside the irradiation field in 7 cases, and cerebrospinal fluid dissemination in 3 cases, which makes the prognosis worse.

Efficacy of bevacizumab for central nervous system radiation necrosis was verified in a randomized double-blind placebo controlled trial in 2011 [7]. Miyatake et al. [8] also demonstrated the usefulness of bevacizumab for radiation necrosis after BNCT. They also showed that radiation necrosis and tumor progression could be differentiated by L/N ratio of the [<sup>18</sup>F]BPA-PET at the cutoff level of 2.0. In the present case, increased enhancement with surrounding edema was diagnosed as radiation

necrosis induced by repeated stereotactic radiosurgery referring to the L/N ratio of the first [<sup>18</sup>F]BPA-PET study. The enhanced lesion and surrounding edema decreased immediately after bevacizumab administration. Since bevacizumab has antitumor effect on uncontrollable meningiomas [9–11], this effect on MR images may have been partly due to tumor regression. After 5 months, the enhanced lesion enlarged again with a remarkable increase in the L/N ratio of the second [<sup>18</sup>F]BPA-PET study, suggesting tumor progression.

Several reports demonstrated histological changes in glioblastoma after BNCT [12–15]. Aziz et al. [12] observed fibrinoid necrosis and vascular hyalinization in 12 glioblastoma autopsy cases at least 4 months after BNCT. Miyatake et al. [15] removed 5 malignant gliomas with increases of enhanced volume in MR images within 3 months after BNCT and histological examination revealed that most of the specimens showed necrosis with small amounts of residual tumor cells of low MIB-1 LI. They concluded that such transient increase in enhanced volume in malignant gliomas and meningiomas immediately after BNCT seemed to be pseudoprogression. Laboratory studies also showed that BNCT induced cell cycle arrest and apoptosis in glioma cells and the MIB-1 LI significantly decreased in the rat glioma model treated with BNCT. These findings suggest that BNCT reduces the proliferative capacity of the tumors [16–18]. To the best of our knowledge, the present study is the first report showing the histological changes in anaplastic meningioma treated with BNCT. The MIB-1 LI was significantly lower in the autopsy specimen obtained 2.5 months after BNCT than that of the specimen obtained at the second surgery for recurrent tumor along with the metastatic liver tumor at autopsy. The therapeutic effect of BNCT at an early stage was therefore pathologically verified.

**Conflict of interest** There is no conflict of interest.

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