

mono- and bis-substitutions of *o*-carboranyl cages to the triazinyl center were further authenticated through X-ray structural studies of **9** and **10**, respectively (Figs. 2 and 3). The crystal structures determined on the basis of X-ray diffraction data corresponded well with the conformations derived from the NMR spectra.

Taking into consideration the three essential requirements for BNCT precursors—good water solubility, low cytotoxicity, and high boron uptake—**9**, **10**, **13**, and **14** appear to be good candidate molecules (see Table 2).⁸ It has been noted that trimelamol shows high cytotoxicity in addition to its high anti-tumor activity. However, four structurally related compounds exhibit low cytotoxicity,¹⁴ with IC_{50} values (the half maximal inhibitory concentration) in the range of 4.49×10^{-5} – 6.54×10^{-5} M. Among the series, A_2B systems were

more soluble in water than AB_2 systems. Furthermore, as the amino functional group was converted to a more polar substituent with a hydroxyethyl group, the water solubility increased to 5.18×10^{-4} (mol/mL) for **13**, which is about two orders of magnitude higher than the solubilities we observed for A_2B -type molecules with alkylamino functional groups in our previous work.¹⁰ All four compounds prepared in the present work (**9**, **10**, **13**, and **14**) were found to accumulate markedly in B-16 melanoma cells when compared to BPA (*p*-boronophenylalanine). We found no direct correlation between water solubility and boron uptake for this series. We attribute the small variation in boron uptake among the four compounds to their similar lipophilic characters, even when a polar functional group was introduced in the A_2B system such as in **13**.

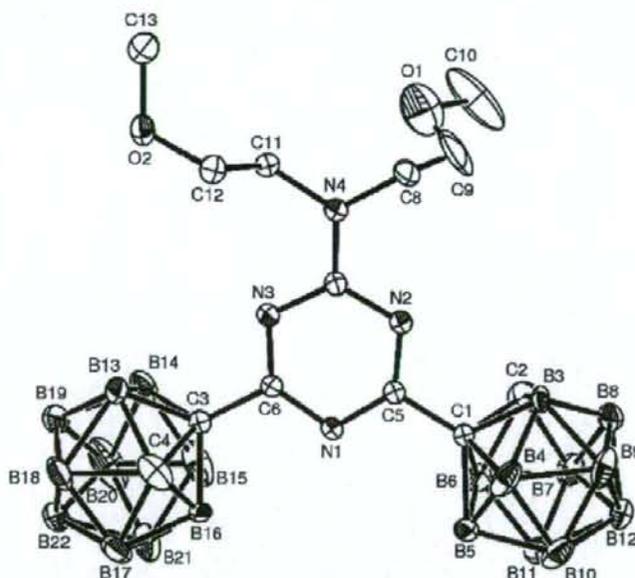


Figure 3. Molecular structure of compound **10**. The thermal ellipsoids are drawn at the 30% probability level.

Table 2. Cytotoxicity (IC_{50}) of the test compounds toward B-16 cells and boron uptake

Compound	B-16 ^a (IC_{50}) M	Boron uptake ^b (μ g B/ 10^6 cells)	Water solubility (mol/mL)
1 9	4.49×10^{-5} (± 0.30)	2.55 ± 0.84	3.27×10^{-6}
2 10	6.54×10^{-5} (± 0.07)	2.01 ± 0.37	1.51×10^{-6}
3 13	4.71×10^{-5} (± 0.33)	1.81 ± 0.81	5.18×10^{-4}
4 14	4.75×10^{-5} (± 0.11)	2.16 ± 2.56	1.03×10^{-5}
BPA	4.49×10^{-5} (± 0.30)	0.083 ± 0.012	

^a B-16: B-16 melanoma cells.

^b Boron uptake by B-16 cells was determined using the ICP-AES method (see Ref. 15). Briefly, cells were cultured in Falcon dishes (90 mm²) until they grew to fill the dishes ($\sim 3.0 \times 10^6$ cells/dish). Cells were then incubated for 3 h with Eagle-MEM medium containing one of the test compounds (boron concentration: 10.8 ppm). After 3 h, the cells were washed three times with PBS(–) and processed for the determination of the boron concentration by ICP-AES. Each experiment was carried out in triplicate.

Electronic supplementary information (ESI) available: experimental details and spectral data for **9**, **10**, **11**, **12**, **13** and **14**. X-ray crystallographic data for **9** and **10** (CCDC No. 655714 and 655715).

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- 6-Chloro-2,4-bis[di(2-methoxyethyl)amino]-1,3,5-triazine (**5**): To a stirred solution of cyanuric chloride **4** (1.84 g, 10 mmol) and *N,N*-diisopropylethylamine (2.58 g, 20 mmol) in 30 mL of THF at -10°C was added di(2-methoxyethyl)amine (2.66 g, 20 mmol) via a syringe. The reaction temperature was maintained at -10°C for 1 h, after which the reaction mixture was warmed slowly to room temperature. The mixture was then stirred for an additional 12 h, after which it was quenched with distilled H₂O (50 mL). The crude product was then extracted with diethyl ether (30 mL \times 2). The organic layer was washed with H₂O, dried with anhydrous Na₂SO₄, and concentrated in vacuo to give 3.62 g (96%) of **5**. Mp $94\text{--}95^{\circ}\text{C}$. ¹H NMR (CDCl₃) δ 3.33 (s, 6H), 3.58 (t, *J* = 5.5 Hz, 4H), 3.86 (t, *J* = 5.5 Hz, 4H). ¹³C NMR (CDCl₃) δ 48.6, 59.0, 70.1, 164.8, 169.9. **Compound 6**: Yield. 91%. Mp $54\text{--}56^{\circ}\text{C}$. ¹H NMR (CDCl₃) δ 3.32 (s, 12H), 3.57 (t, *J* = 5.5 Hz, 8H), 3.85 (t, *J* = 5.5 Hz, 8H). ¹³C NMR (CDCl₃) δ 48.6, 58.9, 70.1, 164.8, 169.9. **Compound 7**: Yield. 96%. Mp $158\text{--}160^{\circ}\text{C}$. ¹H NMR (CDCl₃) δ 1.45 (s, 36H), 4.15 (d, *J* = 45.8 Hz, 8H). ¹³C NMR (CDCl₃) δ 28.11–28.15, 50.0–50.2, 81.9–82.0, 165.4, 168.3–168.5, 169.3. **Compound 8**: Yield. 94%. Mp $65\text{--}66^{\circ}\text{C}$. ¹H NMR (CDCl₃) δ 1.45 (s, 18H), 4.23 (d, *J* = 47.2 Hz, 4H). ¹³C NMR (CDCl₃) δ 28.1, 50.0–50.2, 81.9, 165.4, 168.3–168.5, 169.3.
- 6-(*o*-Carboran-1-yl)-2,4-bis[di(2-methoxyethyl)amino]-1,3,5-triazine (**9**): To a stirred solution of *o*-carborane (1.44 g, 10 mmol) in 30 mL of THF at -78°C was added 2.5 M *n*-BuLi (4.0 mL, 10 mmol) via a syringe. A solution of compound **5** (3.78 g, 10 mmol) in THF was slowly added to the reaction flask at -78°C , and the reaction temperature was maintained at -78°C for 1 h. The reaction mixture was then warmed slowly to room temperature, stirred for an additional 12 h, and quenched with distilled H₂O (30 mL). The crude product was then extracted with diethyl ether (30 mL \times 2). The organic layer was washed with H₂O, dried with anhydrous Na₂SO₄, and concentrated in vacuo. Product **9** was isolated by flash column chromatography (ethylacetate/hexane 1:8) in 18% yield (0.86 g). Mp $97\text{--}98^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2584. ¹H NMR (CDCl₃) δ 3.33 (s, 6H), 3.34 (s, 6H), 3.57 (m, *J* = 5.1 Hz, 8H), 3.84 (m, *J* = 5.1 Hz, 8H), 4.50 (s, 1H). ¹³C NMR (CDCl₃) δ 49.0, 56.1, 59.0, 69.9, 72.7, 163.4, 167.5. **Compound 10**: Yield. 80%. Mp $120\text{--}122^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2563. ¹H NMR (CDCl₃) δ 3.34 (s, 6H), 3.56 (t, *J* = 5.5 Hz, 4H), 3.83 (t, *J* = 5.5 Hz, 4H), 4.41 (s, 1H). ¹³C NMR (CDCl₃) δ 49.0, 56.1, 59.0, 69.9, 72.7, 163.4, 167.5. **Compound 11**: Yield. 12%. Mp $104\text{--}106^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2606, ν (C–H) 1747. ¹H NMR (CDCl₃) δ 1.44 (s, 18H), 1.45 (s, 18H), 4.15 (d, *J* = 45.8 Hz, 8H), 4.43 (s, 1H). ¹³C NMR (CDCl₃) δ 28.1, 50.2–50.5, 56.2, 73.9, 82.1, 164.9, 166.5, 168.4–168.5. **Compound 12**: Yield. 72%. Mp $102\text{--}104^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2582, ν (C–H) 1743. ¹H NMR (CDCl₃) δ 1.46 (s, 18H), 4.24 (s, 4H), 4.36 (s, 1H). ¹³C NMR (CDCl₃) δ 28.1, 51.2, 56.2, 72.4, 83.3, 164.4, 167.2, 167.9.
- 6-(*o*-Carboran-1-yl)-2,4-bis[di(2-hydroxyethyl)amino]-1,3,5-triazine (**13**): To a stirred solution of compound **9** (2.43 g, 5 mmol) in 20 mL of THF at -10°C was added BBr₃ (5.01 g, 20 mmol) via a syringe. The reaction temperature was maintained at -10°C for 30 min, after which the reaction mixture was warmed slowly to room temperature. After stirring for an additional 2 h, the reaction was quenched with distilled H₂O (50 mL). The crude product was extracted with diethyl ether (50 mL \times 2). The organic layer was washed with H₂O and then dried in vacuo. Product **13** was isolated by flash column chromatography (ethylacetate/hexane 1:2) in 54% yield (1.16 g). Mp $106\text{--}107^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2600. ¹H NMR (acetone-*d*₆) δ 3.81 (t, *J* = 5.1 Hz, 8H), 3.87 (t, *J* = 5.7 Hz, 8H), 5.28 (s, 1H). ¹³C NMR (acetone-*d*₆) δ 51.2–51.6, 57.6, 59.0, 73.5, 164.0, 167.3. **Compound 14**: Yield. 71%. Mp $108\text{--}109^{\circ}\text{C}$. IR (KBr pellet, cm⁻¹) ν (B–H) 2600. ¹H NMR (acetone-*d*₆) δ 3.81 (t, *J* = 5.05 Hz, 4H), 3.87 (t, *J* = 5.7 Hz, 4H), 5.28 (s, 1H). ¹³C NMR (acetone-*d*₆) δ 51.2–51.6, 57.6, 59.0, 73.5, 164.0, 167.3.

Compounds 15 and 16: A trifluoroacetic acid (8 mL) solution containing compound **11** (1.41 g, 2 mmol) or **12** (1.22 g, 2 mmol) was stirred for 24 h at room temperature and then dried in vacuo. However, we did not obtain the de-alkylated compounds.

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Synthesis and evaluation of a novel liposome containing BPA-peptide conjugate for BNCT

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Abstract

We aimed at securing sufficient concentrations of ¹⁰B in BNCT by developing a new drug delivery system. We have designed and developed a novel lipid analog and succeeded in using it to develop the new boron component liposome. It consisted of three different kinds of amino acid derivatives and two fatty acids, and could react directly with the peptide synthesized first on resin by Fmoc solid-phase synthesis. In this study, lipid analog conjugated with HIV-TAT peptide (domain of human immunodeficiency virus TAT protein) and boronophenylalanine (BPA) was synthesized and successfully incorporated into liposomes.

Keywords: boron neutron capture therapy (BNCT), boron delivery system (BDS), liposome, boronophenylalanine (BPA), HIV-TAT

1. Introduction

Boron neutron capture therapy (BNCT) is a tumor-selective radiation modality which depends on a sufficient cellular uptake of Boron (¹⁰B) followed by irradiation with a beam of thermal or epithermal neutrons. ⁴He and ⁷Li particles are produced during the neutron capture reaction and damage DNA, which leads to cell killing. Regarding BNCT, the short radiation range of ⁴He and ⁷Li particles is decisive for the distribution of ¹⁰B. Thus, successful treatment of cancer by BNCT requires the selective delivery of relatively large amounts of ¹⁰B compound to malignant cells. The estimated boron concentration required for effective therapy is in the range of 20–30 µg ¹⁰B per g tissue. However there have been no ideal boron compounds that fulfill the conditions of low toxicity, water solubility, and low distribution in normal tissue. Therefore, we aimed at securing sufficient concentrations of ¹⁰B in BNCT by developing a new drug delivery system.

2. Materials and methods

2.1. Synthesis of lipopeptide

Lipopeptide conjugated with HIV-TAT peptide and boronophenylalanine (BPA) was synthesized on TGS-RAM resin by the Fmoc solid-phase synthesis method using an automatic peptide synthesizer (Shimadzu PSSM-8 Peptide Synthesizer Simultaneous Multiple) (Figure 1). Tryptophan residue was added at the N-terminus of HIV-TAT peptide as a fluorescence probe. BPA was

coupled arbitrarily. Then, Fmoc-AEEA (9-fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic acid, linker domain), 11 Fmoc-Asp-OtBu (hydrophilic domain), and Fmoc-Dap(Fmoc)-OH (glycero mimic domain) were coupled sequentially. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), N-hydroxybenzotriazole (HOBt), and N-methyl morpholine (NMM) were used, respectively, for the peptide coupling reaction with 1.0, 1.0, and 1.5 equivalents based on amino acids. Fmoc amino acid and alkyl chain were used for resin in an equivalent of the excess of 7 and 6, respectively. Each coupling reaction was carried out for 30 min. The last condensation reaction with palmitic acid was carried out in a manual mode with the reaction progress checked by a ninhydrin test. De-protection and cleavage of resin were accomplished with a cleavage cocktail (10 mg/mL of 2-methylindole containing trifluoroacetic acid /H₂O/thioanisole/1,2-ethanedithiol/ethylmethyl sulfide/phenol = 82/5/5/3/2/3) for 16 hours at room temperature, then precipitated by adding a large amount of diethyl ether. After the drying procedure, we got a purpose thing.

2.2. Preparation of liposome

The lipid mixture prepared using the constant ratio was dissolved in organic solvent. It was prepared by the

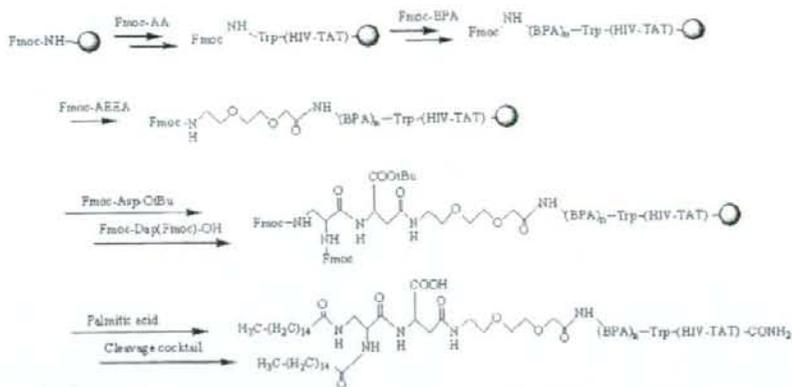


Figure 1. Synthesis of the HIV-TAT and BPA peptide conjugated lipid analog

conventional lipid-film method. The resulting liposomes were extruded through polycarbonate membrane using an extruder, yielding the peptide-modified liposome.

2.3. Gel Filtration Chromatography

The prepared liposome was subjected to size exclusion chromatography, which separated the liposome, micelle and monomolecule fractions, and the content of the lipopeptide was determined (Figure 2). The liposome was then measured using the fluorescence of the tryptophan residue of the lipopeptide.

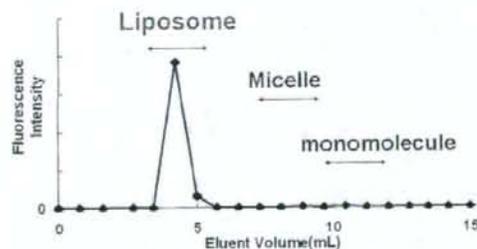


Figure 2. Gel Filtration Chromatography of liposome, micelle and monomolecule

3. Results

3.1. Identification of lipopeptide

In the case of $n=5$ (B5-TAT), the C8-column used for high performance liquid chromatography (HPLC) analysis showed the main peak (retention time at 13.6 min) accompanied by the existence of some impurities (Figure 3a). However, the synthesis of conjugated lipopeptide attached to palmitoyl chain as an anchor domain proceeded very smoothly. HPLC analysis showed almost one peak, and ESI-TOFMS (electrospray ionization

mass spectroscopy) showed m/z 3504 of the dehydration peak as an exact mass of m/z 3522. In addition, ^1H NMR analysis (JEOL JMN-AL400) also showed the structure of the lipopeptide; for example, the molar ratio of the TAT-peptide and palmitoyl moiety showed the correct proton ratio of the ortho position on the tyrosine residue (2H as a characteristic signal of the peptide at 6.62 ppm) to the methyl signal at 0.81 ppm in the alkyl chain end (6H as a characteristic signal of the lipid) using an integration value of ^1H NMR spectra (Figure 3b). The overall yield of the lipopeptide was greater than 70% based on the molar ratio of the amino group on TGS-RAM resin.

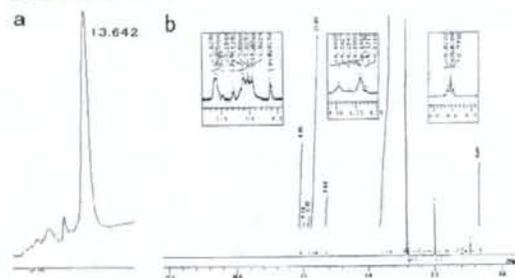


Figure 3. Identification of HIV-TAT peptide and BPA conjugated lipid analog B5-TAT. (a) C8-HPLC, (b) ^1H NMR

3.2. Incorporated ratio of lipopeptide

The incorporation of lipopeptide into the liposome was examined. The methods are showed in Figure 2. Synthesized lipopeptide was incorporated into the liposome effectively. The incorporated ratios of lipopeptide to liposome are summarized in Table 1.

Table 1 Liposome formation and lipopeptide incorporated into liposome

Lipopeptide	Theoretical lipopeptide ratio	incorporated ratio
	(mol %)	(%)
BS-TAT	5	66.1
	10	73.4

4. Discussion

We synthesized a new peptide lipid containing multiple BPA components and a TAT domain for use in a boron-containing liposome which can encapsulate a boron compound in its internal water phase. The peptide lipid can be efficiently incorporated into liposomes that are 100 nm in diameter.

HIV-TAT was first developed from reverse transcriptase of HIV. It is a kind of protein transduction domain³ which can introduce intracellular protein, deoxyribonucleic acid and macromolecular-containing liposome. Yagi *et al.* reported an *in vitro* anti-tumor effect of DOX encapsulated by TAT-modified liposome in 2007.¹ The TAT-conjugating liposome facilitated an *in vitro* gene expression as well as *in vivo* expression when the same liposome was locally injected⁴.

Active targeting against tumor cells using TAT have been evaluated; however, there is no previous report involving a boron-containing TAT liposome or compound.

A sufficient concentration of boron is necessary for successful BNCT. Thus, a material with high boron content generally has an advantage.² Nakamura *et al.* developed a double-stranded boron cluster in 2004.⁵ In the present study, the peptide lipid synthesized contains only 1 to 5 boron in a single molecule. However, our peptide lipid allows the number of boron to be increased up to $n=12$ or $n=15$.

In general, the hydrophilic charge of BSH in a boron-containing liposome has certain difficulty in encapsulating more BSH in the internal water phase of the liposome itself. There has been no previous report involving encapsulated BSH in the internal water phase within a boron liposome. Our peptide modification liposome of the hydrophilic charge is aspartic acid, and it shows high performance in terms of film stability and has a potential advantage in encapsulating BSH in the liposome in which the lipopeptide conjugate BPA.

Further investigation is needed to determine the *in vitro* and *in vivo* toxicity and the boron introduction efficiency.

5. Conclusions

We succeeded in synthesizing a lipopeptide containing boron. This lipopeptide could be incorporated into the liposome effectively. After toxicity testing, these liposomes will be administered to the cells or *in vivo* as a new BDS candidate.

Acknowledgements

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Mini-review

Boron neutron capture therapy for glioblastoma

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Abstract

Boron neutron capture therapy (BNCT) theoretically allows the preferential destruction of tumor cells while sparing the normal tissue, even if the cells have microscopically spread to the surrounding normal brain. The tumor cell-selective irradiation used in this method is dependent on the nuclear reaction between the stable isotope of boron (^{10}B) and thermal neutrons, which release α and ^7Li particles within a limited path length ($\sim 9\ \mu\text{m}$) through the boron neutron capture reaction, $^{10}\text{B}(n, \alpha)^7\text{Li}$. Recent clinical studies of BNCT have focused on high-grade glioma and cutaneous melanoma; however, cerebral metastasis of melanoma, anaplastic meningioma, head and neck tumor, and lung and liver metastasis have been investigated as potential candidates for BNCT. To date, more than 350 high-grade gliomas have been treated in BNCT facilities worldwide. Current clinical BNCT trials for glioblastoma (GBM) have used the epithermal beam at a medically optimized research reactor, and *p*-dihydroxyboryl-phenylalanine (BPA) and/or sulfhydryl borane $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ (BSH) as the boron delivery agent(s). The results from these rather small phase I/II trials for GBM appear to be encouraging, but prospective randomized clinical trials will be needed to confirm the efficacy of this theoretically promising modality. Improved tumor-targeting boron compounds and optimized administration methods, improved boron drug delivery systems, development of a hospital-based neutron source, and/or other combination modalities will enhance the therapeutic effectiveness of BNCT in the future.

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Keywords: Glioma; BNCT; Radiation; Cancer

1. Introduction

The glioblastoma (GBM), a common type of a radio- and chemo-resistant malignant brain tumor in adults, shows rapid tumor growth and wide microscopic invasion to the surrounding normal brain tissue. Despite the improvements in diagnostic modality and the use of intensive multimodal therapies that include surgery, radiotherapy and chemo-

therapy, there have been rather small survival benefits for patients with GBM, which continues to have a median survival time (MST) of less than one year, and even in emerging therapeutic modalities for selected patients, MST is generally less than 2 years. Investigations have revealed the presence of microscopic invading cells at distances of 2–3 cm or even further from the main tumor mass that can be clinically identified by contrast enhancement area on a magnetic resonance image (MRI), and that are found in the microsurgical field during surgical operation. Extensive surgical resection or high-dose

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radiation therapy sufficient to cover microscopic invasion into the healthy brain tissue inevitably leads to some degree of post-therapeutic neurological deterioration. Consequently, 80–90% of GBM recur locally, indicating the need for more intensive and tumor-selective therapy [1–3]. Recently, image-guided surgery utilizing fluorescence with 5-aminolevulinic acid, neuronavigation and intraoperative MRI has enabled more complete resections of contrast-enhancing tumor [4,5]. Concomitant and adjuvant use of temozolomide with a standard photon radiotherapy has demonstrated a significant survival advantage compared to the radiotherapy alone with minimal additional toxicity: the MST was 14.6 months with temozolomide plus radiotherapy and 12.1 months with radiotherapy alone [6].

Several randomized trials have demonstrated a significant improvement of survival time by post-operative fractionated photon radiation at a total dose of 45–60 Gy [7–12]. Among many dose-escalation studies, some small case series found favorable results, which involved dose-escalation mainly in a main tumor mass using an additional stereotactic radiosurgery or other conformal radiotherapy [13–17]. A dose of 90 Gy in accelerated fractionation with photon and proton irradiation almost completely prevented central recurrence, extending the MST of GBM patients treated by this modality to 20 months. However, recurrence occurred in areas immediately peripheral to the 90 Gy volume, mostly in the 70–80 Gy volume, and radiation necrosis also frequently occurred [13]. Therefore, there is urgent need of a method that can deliver high-dose radiotherapy to an extended target area encompassing the microscopic invasion while avoiding radiation necrosis.

Boron neutron capture therapy (BNCT) is the unique high-dose tumor-selective radiotherapy for cancer treatment. BNCT theoretically allows the preferential destruction of ^{10}B -loaded tumor cells, while sparing the normal tissue without ^{10}B , based on the nuclear reaction between ^{10}B and thermal neutrons, which release high linear energy transfer (LET) α and ^7Li particles through the boron neutron capture reaction, $^{10}\text{B}(n,\alpha)^7\text{Li}$ (Fig. 1). The boron neutron capture reaction provide the tumor-selective dose (boron dose), and the other non-selective dose components consist of the proton recoils due to fast neutrons, $^1\text{H}(n,n')p$, 0.54 MeV protons from the nitrogen capture reaction, $^{14}\text{N}(n,p)^{14}\text{C}$, the γ ray arising from contamination in the primary beam, and 2.2 MeV, the

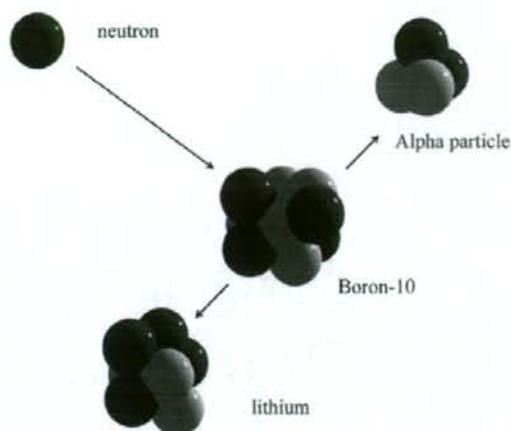


Fig. 1. BNCT is based on the nuclear reaction between ^{10}B and thermal neutrons, which release high linear energy transfer (LET) α and ^7Li particles through the boron neutron capture reaction, $^{10}\text{B}(n,\alpha)^7\text{Li}$. The effectiveness of BNCT is highly dependent on selective boron-10 accumulation in tumor cells as well as sufficient thermal neutrons even at depth.

prompt γ rays from the hydrogen capture. Recent clinical studies of BNCT have focused on high-grade glioma [18] and cutaneous melanoma [19,20], malignant meningioma [21,22], head and neck tumor [23], and lung and liver tumors [24,25] as potential candidates for BNCT. Single session of BNCT seems to be more or at least equally effective as conventional fractionated photon radiation (e.g. 60 Gy by 30 fractions for GBM). However, the procedure is one of the most complex of all anti-tumor treatments and the effectiveness of this therapy is highly dependent on neutron and boron distributions. So far, only a simple-shaped, one direction neutron beam is available in exclusive research reactors. This article provides a review of the clinical trials for BNCT of GBM, and discusses future prospects for this treatment.

2. Clinical studies

2.1. Thermal neutron beam era

The first theoretical account of the biological effects and therapeutic possibilities of BNCT was published by Locher [26]. Early clinical trials of BNCT for brain tumors were conducted by Farr et al. [27,28] at the Brookhaven National Labora-

tory (BNL) and by Sweet et al. [29,30] at the Massachusetts Institute of Technology (MIT) in the 1950s and 1960s. Thermal beams were combined with boron delivery agents, such as borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), *p*-carboxy-phenylboronic acid, and sodium phenyldecaborate ($\text{Na}_2\text{B}_{10}\text{H}_{10}$). To overcome the poor penetrability of thermal neutrons, BNCT was combined with craniotomy to irradiate the brain directly [31]. Neither the early trials at BNL nor those at MIT achieved a significant improvement in survival over the conventional photon beam therapy in use at the time. In their retrospective analysis, Slatkin et al. postulated that the disappointing results may have been due to inadequate numbers of neutrons delivered to the tumor because of the poor penetration property of the thermal neutron beam employed, radiation damage to the cerebral vasculature attributable to the considerable variation of blood boron level among patients, and the insufficient tumor selectivity of boron delivery agent used [32].

In a study by Hatanaka and Nakagawa, more than 200 patients with high-grade glioma were treated with BSH-mediated intraoperative BNCT (IOBNCT). The results showed that the IOBNCT

treatment was more effective than conventional photon beam therapy. The median survival time of GBM and anaplastic astrocytoma (AA) were 22.9 and 64.8 months, respectively. Among the subgroup of 103 high-grade glioma patients, 2 patients with glioblastoma and 4 with AA lived more than 10 years. However, reevaluation of a small subgroup of 12 of the 103 patients according to Curran's recursive partitioning analysis [33] revealed that there were no differences in their survival data compared to those of age-matched controls [34].

2.2. Epithermal neutron beam era; beginning of BNCT without craniotomy

A major advantage of the epithermal beam over the thermal beam relates to the enhanced dose delivery to the deep-seated lesion based on the improved penetrability in the first several centimeters of irradiated volume before epithermal neutrons are moderated and converted into thermal neutrons, generating the $^{10}\text{B}(n,\alpha)$ reaction. The epithermal neutron beam is currently being employed in clinical trials worldwide, as shown in Table 1 [18,35–44]. In a phase I/II clinical trial at BNL and Harvard/MIT,

Table 1
BNCT clinical trials using an epithermal neutron beam for newly-diagnosed GBM

Facility	No. of pts.	Boron drug (infusion)	Normal brain dose peak/ave.	Median survival	Other procedures
BMRR, BNL [18,42,43] 1994–1999	53	BPA: 250–330 mg/kg (2 h)	8.4–14.8/1.8–8.5 Gy(w)	13 M for early 37 pts, 14.8 M for one field group	1–3 fields beam size 10 or 12 cm
MITR-II, MIT [41] 1996–1999	20	BPA: 250–350 mg/kg (1–1.5 h)	8.7–16.4/3.0–7.4 Gy(w)	13 M	1–3 fields beam size 15 cm
JRR-4, JAEA [35] 1998–2004	7	BSH: 100 mg/kg (1 h)	20.3–36.0 ^a /2.3–8.1 Gy(w)	20.7 M	Intraoperative irradiation 1 field beam size 10 or 12 cm
HFR, Petten [36] 1997–	26	BSH: 100 mg/kg/min	8.6–11.4 Gy (boron physical dose)	13.2 M for 10.4 Gy cohort	4 fractions 1 or 2 fields beam size 12 cm
FiR1, Helsinki [39] 1999–	18	BPA: 290–400 mg/kg (2 h)	8–13.5/3–6 Gy(w)	15.0 M	2 fields beam size 11 or 14 cm
Studsvik Medical AB, Sweden [37] 2001–	17	BPA: 900 mg/kg (6 h)	7.3–15.5/3.3–6.1 Gy(w)	18 M	1 or 2 fields
LVR-15, NRI Rez [40] 2001–	5	BSH: 100 mg/kg (1 h)	14.2 >/2 Gy(w)	–	–
KUR, KURRI [38] 2002–	27	BPA: 250–700 mg/kg (1–6 h), BSH: 100 mg/kg (1 h)	15 Gy(w) >/–	–	Local photon radiation or fractionated extended local photon radiation for selected cases
JRR-4, JAEA 2005–	8	BPA: 250 mg/kg (1 h) BSH: 100 mg/kg	13 Gy(w) >/–	–	Fractionated extended local photon radiation (30 Gy)

BNL, Brookhaven National Laboratory; MIT, Massachusetts Institute of Technology; JAEA, Japan Atomic Energy Agency; NRI Rez, Nuclear Research Institute Rez; KURRI, Kyoto University Research Reactor Institute.

^a The peak dose of the tissue adjacent to post-surgical cavity during intraoperative irradiation.

an epithermal beam was used in BNCT to enable an external beam treatment by the closed-cranium method [18]. In these clinical trials, BPA was used as a boron delivery agent. An epithermal beam has also been employed in European clinical trial (EORTC-Study 11961) at the High Flux Reactor (HFR) in Petten that used fractionated closed-cranium BNCT and BSH as a boron delivery agent [36]. Although both trials were primarily phase I safety and dose-escalation studies, the clinical trials at BNL and Harvard/MIT completed in 1999 provided detailed data on normal brain tolerance and survival benefit. MST for the 18 GBMs from the Harvard/MIT trial was 13 months, and was comparable, even as a single session of BNCT, with that of historical controls treated with conventional external beam photon therapy [18,33,41]. The majority of the central nervous system toxicities (CNS) were acute, self-limiting, and primarily related to a temporal increase in intracranial pressure. A tumor volume of more than 60 cm³ was associated with a higher incidence of grade 3–4 symptoms. In the BNL dose-escalation study, the MST for the first 38 of 53 subjects was 13 months. However, retrospective analysis revealed that the later subgroup, which showed an increased average brain dose (ABD) and an increased number of irradiation fields, had a higher incidence of CNS toxicity and did not compare favorably with the historical controls. Median times to progression from histopathological diagnosis following one, two and three field BNCT were 34.5, 28.1, and 18 weeks, respectively. MST following one, two and three field BNCT were 14.8, 12.1, and 11.9 months, respectively. Two of the seven subjects received an ABD of 8 Gy-Eq or above, using three fields, and had grade 3 CNS toxicity [42]. An ABD of 6.2 Gy-Eq was associated with 50% incidence of somnolence [43]. In the EORTC-Study 11961, BNCT was carried out in 4 fractions on 4 consecutive days and BSH were administered enough to keep the average boron concentration over the 4 fractions at 30 ppm. Beginning at the starting dose at 8.6 Gy as boron dose, four dose-escalation cohort were treated. A single field with 12 cm collimator was used for the first 5 patients and 2 fields were used for later subjects. A better MST of 13.2 months was recorded in the third dose-escalation cohort [36].

The clinical teams at the Japan Research Reactor-4 (JRR-4) employed an epithermal neutron beam into the BSH-mediated IOBNCT [35,44,45]. Among 16 newly diagnosed GBMs treated by the

clinical team at the University of Tokushima and the University of Tsukuba, 2 subjects were irradiated with a pure epithermal beam and 14 subjects were irradiated with a mixed thermal-epithermal beam at 12 h following the 1 h intravenous infusion of BSH (250 mg/kg). Single field and either a 10 or a 12 cm-collimator was minimized by an LiF shielding device at the craniotomy window, resulting in a relatively low ABD of approximately 5 Gy-Eq. In 7 GBMs from the University of Tsukuba underwent IOBNCT, and the MST and the median PFS for the small series were 22.6 months and 12.0 month, respectively [35].

2.3. Modification of BNCT with an epithermal neutron beam

Since the addition of epithermal neutron beam irradiation to BNCT for the treatment of high-grade gliomas, a sufficient number of thermal neutrons have been delivered to most unilateral tumors. However, once the tumor volume reduction on MRI was achieved, local recurrences occurred in the majority of the subjects treated with the external beam BNCT, while both local and distant recurrence occurred in the subjects receiving IOBNCT [35,36,42]. These observations imply that the boron concentrations in the infiltrating tumor and the main tumor mass are insufficient and heterogeneous to a certain degree. Therefore, the main key to successful BNCT for GBMs is a homogeneous preferential accumulation of boron-10 in the main tumor mass as well as in the infiltrating tumor cells. Preclinical data suggest that a longer infusion time may improve this situation [46–48]. In an *in vitro* study, the accumulation of BPA appeared to be more cell-cycle dependent than the accumulation of BSH, but cell-cycle dependency in the accumulation of BPA became minimal over the long-term infusion [48]. In the *in vivo* rat 9L gliosarcoma model, up to 6-h infusion of BPA increased the tumor boron-10 concentration, maintaining a constant tumor-to-blood ratio of 3.7:1 [46]. In a quantitative microlocalization study, extending the time of BPA administration improved the B-10 distribution, with 6-h intravenous infusion of BPA at a dose rate of 250 mg/kg/h resulting in a nearly 90% higher boron-10 concentration in infiltrating tumor cells compared to 2- and 3-h infusions [47].

The long-term infusion method has been applied in the Swedish clinical trial since 2001. BPA administration at a dose of 900 mg/kg over 6 h has been

well tolerated, and the interim median survival time for the first 17 GBMs was 18 months [37]. In this protocol, neutron irradiation has been performed using 2-fields and an average brain dose (ABD) ranging from 3 to 6 Gy(w).

The combination of BPA and BSH was first applied to clinical use by Miyatake and associates with the intention of minimizing the heterogeneous boron-10 distribution based on the different mechanisms by which BPA and BSH accumulate in tumor cells [38]. In principle, ^{18}F -labeled positron emission tomography (PET) was followed by BNCT to calculate the L/N ratio of BPA-mediated boron-10. BSH (5 g/body) and BPA (250 mg/kg) were administered intravenously 12 and 1 h before epidermal neutron beam irradiation, respectively. Regardless of the pre-BNCT tumor volume, improvements on neuroimages were recognized both at the initial (46.4%) and at follow-up (58.5%) assessments. More than 50% of the contrast-enhanced lesions disappeared in 8 of the 13 patients during the follow-up period. In the following series, the total amount of BPA was increased to 700 mg/kg, infused over 6 h to yield a more homogeneous boron distribution.

In the Finnish trial conducted by Helsinki University Central Hospital (HUCH) and the Technical Research Center of Finland (VTT), 290 mg/kg of BPA infused over 2 h was used in the first 12 GBMs with two irradiation fields [39]. The BPA dose to subsequent GBMs was escalated from 330 ($n=1$) to 360 mg/kg ($n=3$), 400 ($n=3$), and 450 mg/kg ($n=3$). These BPA doses with a planning target volume (PTV) dose ranging from 30 to 72 Gy(w) achieved a median survival of 15 months with no dose-limiting toxicity encountered. Moreover, the 7 GBMs that recurred after conventional photon irradiation of 50–60 Gy showed a favorable response to reirradiation with BNCT using 290 mg/kg of BPA infused over 2 h, in which protocol the PTV dose ranged from 25 to 36 Gy(w) and the average whole brain dose ranged from 2 to 4 Gy(w).

Although a number of studies have reported on the efficacy of multimodal treatment for brain tumors, there have been relatively few investigations on combinations between BNCT and other modalities. Because of the relatively low normal brain dose, BNCT allows additional photon radiation sufficient to maintain a total normal brain dose below the tolerance limit. An *in vivo* study revealed that a photon radiation boost after BNCT could significantly enhance the survival of F98 glioma- and

MRA 27 melanoma-bearing rats after administration of BPA and BSH [49]. Clinically, the photon boost has been used in recent protocols in order to ensure a sufficient BNCT dose to deep-seated lesions [38]. Since 2005, new protocol with a combination of fractionated, extended local photon radiation and BPA- and BSH-mediated BNCT was begun at JRR-4. In this protocol, boron-unloaded subpopulations of tumor cells are irradiated only by non-selective components of BNCT radiations and fractionated photon radiation, and an additional boron dose boosts these non-selective doses in the boron-loaded tumor cells. Eight patients with newly diagnosed GBMs were followed up for 17.2 months (mean) after treated by this protocol, six patients are alive at analysis and the MST and the median PFS for the patients are 19.8 and 12.0 months, respectively (unpublished data).

2.4. Optimization of BNCT

Successful BNCT is highly dependent on the selective accumulation of boron-10 in tumor cells. There have been only two boron delivery agents available for clinical BNCT trials for high-grade glioma (Fig. 2), *p*-dihydroxyboryl-phenylalanine (BPA) and the sulfhydryl borane $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ (BSH). BSH biodistribution studies have suggested that the primary mode of the selective BSH distribution is passive diffusion from blood to tumor tissue *via* the disrupted blood–brain barrier (BBB) [50,51]. The boron concentration in the normal brain with an intact BBB remains minimal, while the tumor boron-10 concentration is related to both the tumor vascularity and the blood boron-10 level. The tumor-to-blood boron concentration ratio ranged from 0.5 to 1.0 in the biodistribution studies using animal brain tumor models [52,53]. Tumor-to-blood boron concentration ratios of 0.5–1.0—a slightly

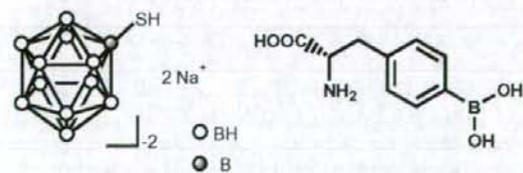


Fig. 2. Currently available boron delivery agent for BNCT. Left: sulfhydryl borane $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ (BSH); right: *p*-dihydroxyboryl-phenylalanine (BPA).

higher range than in these animal studies—have been reported in human patients treated with BSH-mediated BNCT [51].

BPA, the amino acid analogue, has structural characteristics similar to those of a melanin precursor. The findings that BPA is selectively accumulated *in vivo* and *in vitro* and that it achieves a high tumor control rate after neutron irradiation support the potential application of BPA for clinical BNCT of melanoma. Mishima et al. reported promising clinical results in a pilot study of BNCT for skin melanoma [19,20,54]. In contrast to BSH, BPA is actively transported through the tumor cell membrane due to elevated rate of amino acid transport in proliferating cells [48,55]. Non-proliferating cells escape radiation damage following BNCT. The recent clinical trials have used a combination of BPA and BSH with the intention that these different compounds would accumulate in different sub-populations of tumor cells.

To date, a variety of boron delivery agents have been investigated, including porphyrins [56,57], amino acids [58], polyhedral borane [59], carbohydrates [60], polyamines [61], biochemical precursors [62,63], DNA-binding agents [64,65], antisense agents [66], peptides [67], liposomes [68–70], and monoclonal antibodies [71]. A boron delivery agent for BNCT would be required to fulfill all of the following conditions: (a) non-toxic at a dose of clinically use (b) to achieve at least 10–30 $\mu\text{g } ^{10}\text{B/g}$ of tumor boron-10; (c) high tumor/brain and tumor/blood concentration ratios; (d) rapid clearance from blood circulation and normal tissues but persistence in tumor; (e) water solubility; and (f) chemical stability [49,72]. Among the boron delivery agents, BOPP [73], a boronated porphyrin, and GB-10 [59], polyhedral borane $\text{Na}_2\text{B}_{10}\text{H}_{10}$, have been evaluated for their pharmacokinetics in humans. A phase I trial for potential application of BOPP for photodynamic therapy was interrupted because of its general toxicity [73]. These results suggest the need for development of a better boron delivery agent, as well as an improved method of drug delivery. Recently, improvement of the synthesis pathway, and novel agents such as porphyrin-cobaltacarborane conjugates [74] or ^{10}B -enriched carboranyl-containing phthalocyanine [75] have been under examination. GB-10 has been proposed as a boron neutron capture agent for an additional dose in fast neutron therapy. In a trial in which GB-10 was used in 15 lung cancer patients, no toxicity was recorded [59].

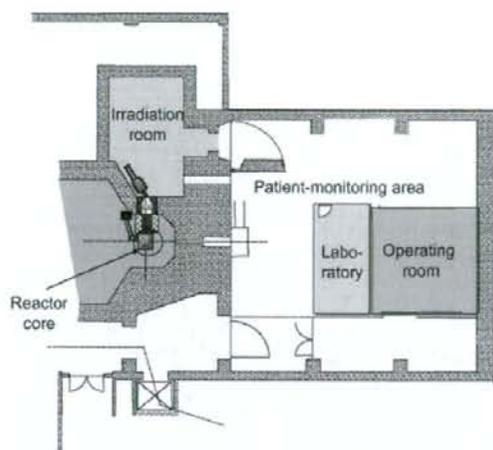


Fig. 3. The general arrangement of the medical irradiation facility of Japan Research Reactor-4, showing the irradiation room located in the basement.

The use of epithermal neutron beams has provided a non-invasive method for clinical BNCT without craniotomy for high-grade glioma, and the encouraging results have motivated further investigations to optimize this unique therapeutic modality. At the present time, clinical BNCT trials have been performed at limited numbers of nuclear reactors that have simple medical facilities (Fig. 3). A prompt gamma-ray analysis (PGA) device, an inductively coupled plasma atomic emission spectroscopy (ICP-AES) device for boron-10 measurement, and an irradiation room are installed in the reactor for BNCT [35]. For dose planning of BNCT, ^{18}F -labeled PET has been employed to calculate the L/N ratio of BPA-mediated boron-10 in some institutions [38,76]. However, because patients must be transferred from and returned to medical centers at varying distances from the reactor facilities, there is need for a hospital-based compact reactor or accelerator-based neutron source [77] that could be constructed in a selected medical center specializing in the treatment of brain tumors.

3. Conclusion

BNCT is an emerging therapeutic modality for cancers which can theoretically allow tumor-selective destruction while sparing normal tissue. The selectivity is highly dependent on the dose from the boron neutron capture reaction, $^{10}\text{B}(n, \alpha) ^7\text{Li}$, i.e., the accumulation of boron-10 in tumor cells.

Recent clinical studies of BNCT have focused on the treatment of high-grade gliomas and cutaneous melanomas. More than 350 high-grade gliomas have been treated in BNCT facilities worldwide. Cerebral metastasis of melanoma, anaplastic meningioma, head and neck tumor, and lung and liver metastasis have been investigated as potential candidates for BNCT [21–23]. In the near future, ongoing clinical BNCT trials will disclose the dose-related effectiveness and safety of BNCT, the most suitable method of administering boron drugs, and the feasibility of combination with photon therapy. The clinical results from the rather small trials performed to date appear to be encouraging. In the future, improvement of the tumor-targeting boron compound, the administration method, and the drug-delivery system, and development of a hospital-based neutron source and/or other combination modalities will enhance the therapeutic effectiveness of BNCT.

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Combined use of sodium borocaptate and buthionine sulfoximine in boron neutron capture therapy enhanced tissue boron uptake and delayed tumor growth in a rat subcutaneous tumor model

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Abstract

We have previously reported that buthionine sulfoximine (BSO) enhances sodium borocaptate (BSH) uptake by down regulating glutathione (GSH) synthesis in cultured cells. This study investigated the influence of BSO on tissue BSH uptake *in vivo* and the efficacy of BSH–BSO-mediated boron neutron capture therapy (BNCT) on tumor growth using a Fisher-344 rat subcutaneous tumor model. With BSO supplementation, boron uptake in subcutaneous tumor, blood, skin, muscle, liver, and kidney was significantly enhanced and maintained for 12 h. Tumor growth was significantly delayed by using BSO. With further improvement in experimental conditions, radiation exposure time, together with radiation damage to normal tissues, could be reduced.

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Keywords: Boron neutron capture therapy; Sodium borocaptate; Buthionine sulfoximine; Glutathione; Boron uptake; Rat tumor model

1. Introduction

Boron neutron capture therapy (BNCT) is a bimodal radiation therapy in which a neutron cap-

ture agent, a stable isotope of boron (¹⁰B), is preferentially delivered into the malignant tissues by using a boron delivery compound, followed by irradiation with a beam of low-energy (thermal or epithermal) neutrons. Differential ¹⁰B concentrations between the malignant tissues and the surrounding healthy tissues enable selective destruction of malignant tissues. But in practice, efficacy of BNCT is limited by

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the number of available ^{10}B atoms in tumor due to lack of selectivity for tumor by boron delivery compounds [1,2].

Two known boron compounds, boronophenylalanine (BPA) and sodium borocaptate (BSH) are currently used, sometimes in combination, as boron delivery agents. BPA is preferentially taken up by growing tumor cells, whereas BSH is known to preferentially accumulate in malignant brain cells by penetration into tumor-induced disrupted blood–brain barrier [3,4]. In comparison with BPA, the total amount of BSH uptake was less and its uptake has little influence on the cell cycle [5].

We have shown in our previous *in vitro* study that buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, when given together with BSH, depletes intracellular glutathione (GSH) by inhibiting GSH biosynthetic enzyme γ -glutamylcysteine synthetase and consequently enhances BSH uptake and cytotoxic effect [6].

In this study, we attempted to validate the influence of BSO on tissue uptake of BSH and to investigate the efficacy of combined BSH–BSO treatment on tumor growth by using a rat tumor model.

2. Materials and methods

All experimental animal protocols were pre-approved by the Animal Experiment Committee of University of Tsukuba and conducted following “the Regulation for Animal Experiment” guidelines.

2.1. Tumor cell line

Rat gliosarcoma cell line (9L) was maintained in Earl's minimal essential medium, MEM, (Sigma–Aldrich, Japan) supplemented with 10% fetal bovine serum.

2.2. Reagents

Reagents used (and vendors) were as follows: BSH (Katchem, Czech Republic), BSO and tribromoethanol (Sigma–Aldrich, Japan), isoflurane (Foren, Abbott, Japan), and standard boron solution (Wako Pure Chemicals, Japan).

2.3. Animal experimentation and protocol

For determination of tissue boron concentration, 7-week-old male Fisher 344 rats (weighing ca. 150 g) were used, whereas for neutron irradiation experiments, due to size limitation by the unique design of our custom-made irradiation chamber (Fig. 1), 3-week-old male Fisher 344 rats (weighing ca. 35 g) were used.

Approximately, 10^6 of 9L cells (100 μl) were inoculated subcutaneously into right leg of animals. Two weeks later, when a subcutaneous tumor developed into a diameter of ca. 7 mm, tumor-bearing animals were randomly divided into three groups: (a) BSO+ group received BSO (5 mmol/kg) in 3 ml phosphate buffered-saline (PBS) *i.p.* and BSH (100 mg/kg) in 300 μl PBS *i.v.* via tail vein; (b) BSO– group received BSH (100 mg/kg) in 300 μl PBS *i.v.* via tail vein; and (c) untreated controls. The procedure was done under isoflurane anesthesia.

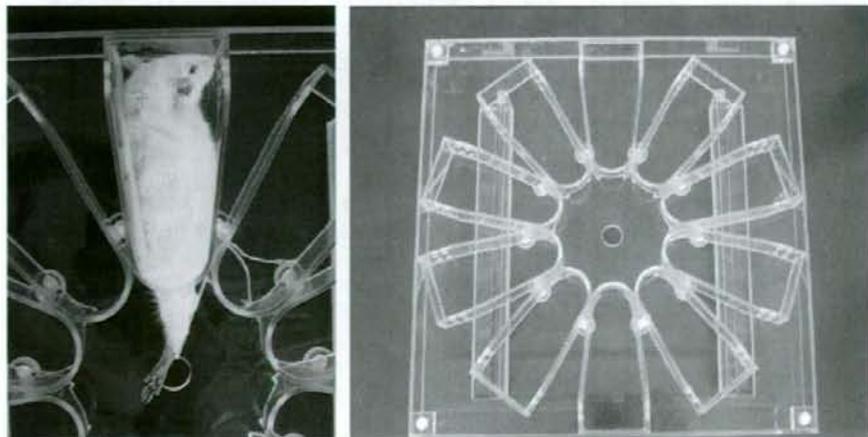


Fig. 1. Neutron irradiation of a rat with a subcutaneous tumor on the right leg using the custom-made irradiation chamber.

To examine tissue boron distribution, the animals in the BSO+ and BSO- groups ($n = 8$ each) were sacrificed at 3, 6, and 12 h after the administration of drugs, and tissue samples from subcutaneous tumor, blood, brain, skin, muscle, liver, and kidney were obtained.

To observe tumor growth after BNCT, the animals in the BSO+, BSO-, and control groups ($n = 5$ each) were irradiated at 6 h after the drug administration.

2.4. Measurement of tissue boron concentration by inductively coupled plasma-atomic emission spectroscopy (ICP-AES)

The tissues samples were liquefied by thermolysis with 20–30% nitric acid at 115 °C for 2–3 h using a thermoalumi-bath (ALB-121, ASAHI TECHNO GLASS, Tokyo) and water was added to a final volume of 5 ml, followed by filtration through a membrane filter (PTFE 0.5 μm , Toyo Roshi Company, Ltd., Japan). The filtrate was measured for its boron content by ICP-AES (Jarrell-Ash ICAP-575, Nippon Jarrell-Ash Co., Ltd., Kyoto, Japan). The boron concentration was determined using a calibration curve of standard boron solution (Wako Pure Chemicals, Japan).

2.5. Neutron irradiation

Neutron irradiation experiments were carried out at the JRR-4 research reactor in the Japan Atomic Energy Agency (JAEA). Tumor-bearing animals were irradiated using a custom-made irradiation chamber designed to accommodate 10 rats that can be fixed on a plexiglass board and irradiated simultaneously (Fig. 1). The irradiation was performed using a ϕ 10-cm collimator in a thermal neutron flux of 3.7×10^8 n/cm²/s for 60 min. Thermal neutron fluence was measured using gold wire activation. Rat anesthesia was induced with tribromoethanol (500 mg/kg/i.p.) and maintained by isoflurane inhalation during the irradiation. The absorbed dose was calculated using the following formula described previously [7]:

$D_{\text{total}} = D_n + D_b + D_f + D_\gamma$ (where D_n = the absorbed dose due to the nitrogen capture reaction, D_b = boron capture reaction dose, D_f = the fast neutron dose, and D_γ = the γ -ray dose. The relative biological effectiveness (RBE) for nitrogen capture reaction and boron capture reaction used was 1.9 and 2.3, respectively [8].

2.6. Monitoring tumor growth

After the neutron irradiation, tumor size (i.e., tumor volume in mm) was measured for 3 weeks at days 0, 3, 6, 10, 14, 17, and 20, using the following formula:

$$\text{Tumor volume} = (\text{minor axis})^2 \times \text{major axis} / 2$$

2.7. Statistical analysis

Data were analyzed with *U* test of Mann-Whitney and probability values of <0.05 were considered significant.

3. Results

3.1. Tissue boron concentration

Table 1 shows the effect of BSO on the boron distribution in different tissues. Regardless of BSO supplementation, tissue boron concentrations (for both the BSO+ and BSO- groups) peaked at 3 h after the administration of BSH with or without BSO and gradually decreased with time. In comparison with the BSO- group, the boron concentration in all tissue samples, except the brain sample at 6 h and 12 h, was significantly higher in the BSO+ group ($p < 0.001$ – 0.05).

3.2. Tumor growth after BNCT

As shown in Fig. 2, as early as day 10 after the neutron irradiation, there were obvious differences in tumor growth as determined by tumor volume. Specifically, a significant delay in tumor growth was observed in the BSO+ group as compared with the control group (p -value at days 10, 14, 17 was 0.02, 0.01, and 0.04, respectively). There were also noticeable, albeit not significant, differences in tumor growth between the BSO+ and BSO- groups as well as the BSO- and the control group.

4. Discussion

4.1. Enhanced tissue boron uptake by BSH–BSO combination

GSH, predominant intracellular non-protein thiol compound, is involved in many biological functions including antioxidation, detoxification, and protection against peroxidation, UV, heavy metals, many exogenous organic substances such as anticancer agents, and radiation damage [9,10]. The most important physiological role of GSH with regard to BNCT is radiation protection, which is considered to be through direct radical scavenging or donation of reducing equivalents to terminate a free radical reaction [11].

The dose-dependent manner of BSO-induced intracellular GSH depletion has been documented in the normal and tumor cells [6,12,13]. A range of BSO doses (0.5–5.0 mmol/kg) have been used in the treatment of various tumors [12–15]. We used 5 mmol/kg in our studies because this dose has been shown to achieve the maximum GSH depletion in

Table 1
Tissue boron concentration ($\mu\text{g/g}$) of 7-week-old Fisher-344 rats ($n = 8$)

	3 h ^a			6 h ^a			12 h ^a		
	BSO+	BSO-	<i>p</i> -Value ^b	BSO+	BSO-	<i>p</i> -Value ^b	BSO+	BSO-	<i>p</i> -Value ^b
Subcutaneous tumor	26.3 ± 4.3	5.0 ± 2.9	0.0027	11.9 ± 7.8	1.3 ± 0.6	0.0019	6.5 ± 9.9	1.0 ± 0.1	0.0009
Blood	55.2 ± 15.0	8.1 ± 6.2	0.0027	20.2 ± 14.0	3.4 ± 0.5	0.0055	10.8 ± 20.6	1.3 ± 0.2	0.0005
Brain	2.6 ± 1.4	0.6 ± 0.7	0.0101	0.7 ± 0.6	0.2 ± 0.1	0.1967	0.3 ± 0.4	0.1 ± 0.1	0.2004
Skin	34.2 ± 9.8	6.3 ± 4.3	0.0027	22.1 ± 13.9	3.6 ± 0.9	0.0012	9.7 ± 13.4	1.8 ± 0.3	0.0017
Muscle	7.4 ± 2.8	1.2 ± 0.9	0.0027	5.0 ± 3.6	0.8 ± 0.2	0.0321	0.3 ± 0.4	0.1 ± 0.0	0.0007
Liver	71.4 ± 22.6	12.6 ± 8.1	0.0027	30.0 ± 18.8	9.4 ± 1.9	0.0109	4.6 ± 7.6	0.8 ± 0.1	0.0005
Kidney	82.3 ± 28.5	31.6 ± 15.9	0.0043	42.2 ± 16.5	24.1 ± 4.0	0.0109	6.8 ± 7.3	2.4 ± 0.5	0.0054

Data are shown as mean ± SD.

^a Time after the administration of BSH with or without BSO.

^b *p*-Values account for the difference in the boron concentration between the BSO+ and BSO- groups.

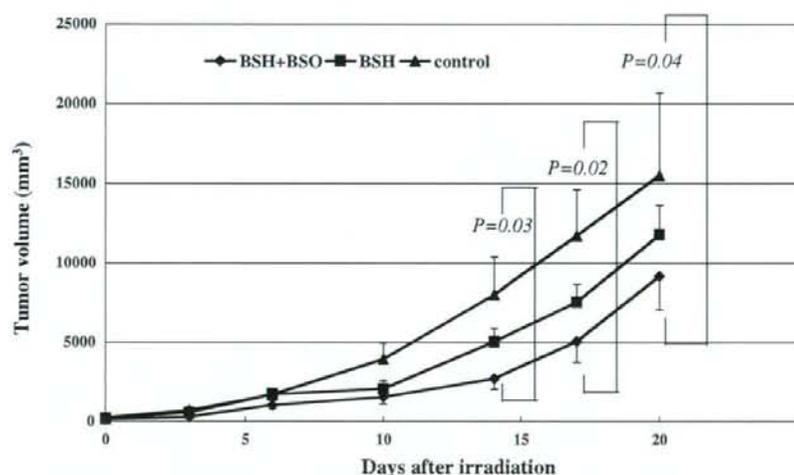


Fig. 2. Tumor growth curve of Fisher-344 rats after the BSH-mediated BNCT with and without BSO ($n = 5$, error bars = SE). *p*-Values account for the differences between the BSO+ and the control groups at specific time points. See Section 2 for experimental details.

tumor cells with no apparent side effects in mice [12,13].

We showed in our previous study that by depleting GSH, BSO resulted in an increased BSH uptake with associated radiosensitization and reduction of radiation damage to the cultured cells [6]. In this in vivo study, boron concentrations in tumor tissue, blood, skin, muscle, liver, and kidney were enhanced significantly (e.g., nine times in the subcutaneous tumor at 6 h) and maintained at high level for as long as 12 h after the administration of BSH and BSO. The observed sustained and high tissue boron levels is likely due to the formation of borocaptate dimer (BSSB), because the retention of the BSSB in cultured cells has been shown longer compared to that of the monomer BSH [16–20].

Clendenon et al. [21] previously demonstrated that the blood boron concentration in non-tumor-bearing rats decreased 4.8 times from 38.8 $\mu\text{g/ml}$ at 0.5 h to 8.0 $\mu\text{g/ml}$ at 3 h after the administration of BSH. In this study, the blood boron concentration in the BSO- group of 8.1 $\mu\text{g/ml}$ at 3 h, which is parallel to Clendenon's data, was enhanced 6.8 times to 55.2 $\mu\text{g/ml}$ in the BSO+ group. It is therefore conceivable that in the presence of cellular GSH, the significant loss of the administered BSH occurs within 3 h and depletion of cellular GSH by BSO averts the loss. Besides, as shown in Table 1, large SD values for the tissue boron concentrations indicate great variation within the group. In the BSO+ group, the animals with higher blood boron concentration