

M. Suzuki

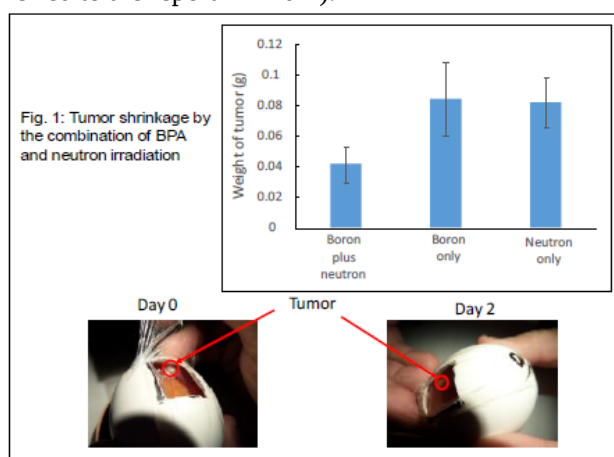
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Summary

In this research project, fifteen research project were included. Gadolinium-containing compounds were studied in two studies (P10-7 and P10-12). In other thirteen studies, boron-containing compounds were studied. New boron drugs studied in this project consist of eight small boron molecular agents and five nanoparticles.

In the fifteen projects, new compounds in ten projects were tested on efficacy of tumor control or cell killing using neutron irradiations.

In five research project (P10-8, P10-10, P10-13, P10-15 and P10-16), in-vivo study using tumor-implanted mice or rats were carried out. In one project (P10-4), tumor-implanted egg was interestingly used as shown in the below figure (the detail is referred to the report in P10-4).



In one research projects (P10-14), in-vitro study with neutron irradiation was carried out. In two projects (P10-5, P10-9), a pharmacokinetic studies of new boron compounds were done.

In vivo study

The results in five in-vivo studies using tumor-bearing mice or rat studies are summarized as bellow.

P10-8: Two studies using two new boron compounds were done. Study 1: A significant prolongation of median survival time (MST) in survival time in boronophenylalanine (BPA) and novel pteroyl clo-

so-dodecaborate conjugate (PBC)-BNCT group was obtained compared with the other single agent (BPA or PBC)-BNCT group.

Study 2: Sensitization of the 5-aminolevulinic acid (ALA) in BPA-BNCT was examined. The mice in the BPA-BNCT with ALA group obtained a significant prolongation in survival time compared with BPA-BNCT group.

P10-10: New boron compounds, BN1229 and BN1242 were tested in this research. A significant tumor growth inhibitory effect was observed in BN1242 group compared with the untreated control.

P10-13: Complex of BPA and poly vinyl alcohol (PVA) which has many BPA molecules was tested. PVA-BPA-BNCT exhibited drastic antitumor activity and tumor regrowth was not observed even at day 18.

P10-15: Novel liposome modified with novel lipid (name PBL) as boron delivery system (BDS) was tested. The PBL-liposome and borocaptate sodium (BSH)-encapsulated PBL-liposome significantly inhibited the tumor growth as compared to other control groups.

P10-16: Novel boron compound, BNC2018 was tested. The tumor growth suppression effects derived the highly tumor retnivity of BN2018 by BNCT.

In vitro study

The results in one in-vitro studies using neutron irradiation are summarized as bellow.

P10-14: A novel boron compound, PBC, was tested. PBC demonstrated their cytotoxicity with neutron irradiation.

Other new boron or gadolinium compounds which were not tested with neutron irradiation will be expected to be examined in 2018-2019.

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Despite extensive efforts, it is difficult to ensure that the selective targeting of ¹⁰B will be successful. One can argue whether Locher's BNCT theory feasible? Scientists have made extensive effort to answer this question and to discuss the potential future problems of BNCT. From the viewpoint of boron chemistry, the conditions required for boron/gadolinium targeting are: (1) a low toxicity, (2) the ability to be held in a tumor and/or tumor cells selectively for a certain period of time, (3) to be rapidly excreted from the body system. These points are essential, but to achieve each of these at the same time is a very difficult task.

We have investigated the theranostic (therapeutic+diagnostic) agent exhibiting cellular quantitative distribution in *in-vitro* using a fluorescent density microscope without any neutron irradiation. Also, fluorescent chemical substances have the potential to be utilized for intraoperative probing of the tumor by using fluorescent surgical microscopy. Two carboranes conjugating-ligands have been synthesized and the properties of fluorescent sensors for boron derivatives are reported. This appears to be a useful method for the screening of boron compounds without neutrons.

Fluorescein was conjugated with 1-methyl-o-carborane (**Figure 1**) via a Williamson ether synthesis and biologically evaluated through studies in pancreatic cancer (MIA PaCa-2) and squamous cell carcinoma (SCC-VII) cell lines. Cellular uptake was confirmed through phase contrast, fluorescent, and confocal microscopy, as well as flow cytometric data. Uniform distribution of the carboranyl-fluorescein derivative was observed in both SCC-VII (**Figure 2**) and MIA PaCa-2 (**Figure 3**). The cytotoxicity of fluorescein-tagged 1-methyl-o-carborane in SCC-VII was determined using a WST-8 assay. An IC₅₀ of 1 mM or 21 ppm ¹⁰B was observed, reflecting moderate toxicity relative to that of BSH

(28 mM) and BPA (7.9 mM). This moderate cytotoxicity and uniform distribution of the carboranyl-fluorescein derivative following cellular uptake in both SCC-VII and MIA PaCa-2, as well as its higher boron content relative to boronated agents currently employed in clinical trials, provide justification for further evaluation as a potential delivery agent for BNCT. Biodistribution studies and survival assays are scheduled at IIRNS of Kyoto University on this FY2018.

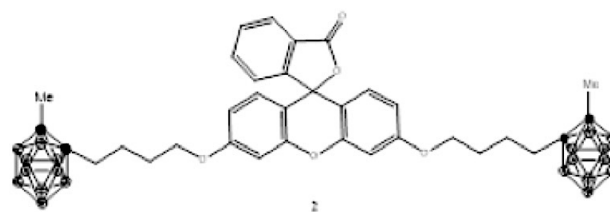


Figure 1: The molecular structure of fluorescein-tagged 1-methyl-o-carborane.

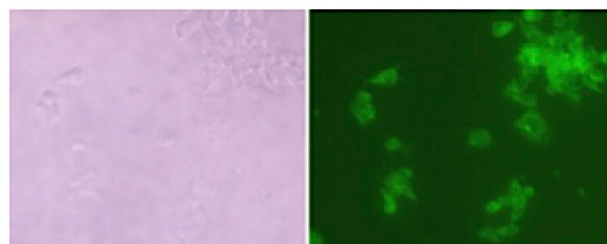


Figure 2. The microdistribution of carboranyl-fluorescein conjugate 1 in SCC-VII cells. (A) Light microscopic image of SCC-VII cells treated with compound 1. (B) Fluorescent microscopic image of SCC-VII treated with compound 1.

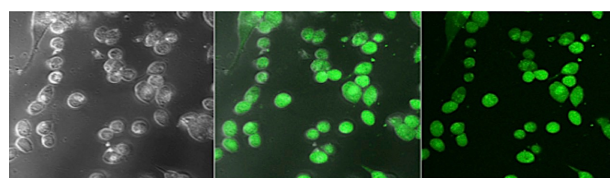


Figure 3. Fluorescent microscopic images of MIA PaCa-2 cells containing a 40 µg/mL sample of carboranyl-fluorescein conjugate 1 in DMSO: (A) black and white filter, (B) merged black/white and green filter, and (C) green filter.

PR10-2 New Self-assembling Peptide Drug Delivery System with BSH against Human Glioblastoma Cell in BNCT

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INTRODUCTION: Glioblastoma multiforme (GBM) is the most common malignant central nervous system primary tumor, and not curable. BNCT (boron neutron capture therapy) is the effective treatment against GBM in present multimodal therapy. In BNCT in GBM, one of the keys to success can depend on the boron compounds. The adequate boron delivery into all of every tumor cells is essential for BNCT to GBM. The combination of BSH and BPA in clinical GBM BNCT showed very good results and that meant the multi boron use in BNCT was one answer to next step of BNCT. In this time, we showed results of the new self-assembling peptide DDS with BSH toward clinical application.

EXPERIMENTS: We established the simple A6K/BSH complex making method, as just mixture the BSH and A6K water solution by itself. The BSH/A6K complex with different mixture ratio showed different shape and different diameter of complex in SEM image. We decided the particular mixture ratio of A6K/BSH, 1:10 mol ratio, complex as the most fitted for drug delivery system to brain tumor. The ideal range of particle size of DDS is 20nm to 200 nm, and ours' complex diameter was about 40nm. Next, we administrated BSH/A6K complex to human glioma cell lines and measured intracellular boron uptake. The intracellular boron concentration with BSH/A6K complex in U87 delta EGFR was 10 times higher than that with BSH. We reconfirmed the particular mixture ratio of BSH/A6K, 1:10 complex as the most fitted for drug delivery system to brain tumor. Finally, we administrated BSH or A6K/BSH complex to glioma cells and irradiated neutron *in vitro*.

RESULTS: As shown in Fig. 1, WST-1 assays showed that the group of A6K/BSH complex and, 15min or 30min inhibited U87 DELTA EGFR proliferation. On the

other hand, only BSH administration group with 15min and 30min.

CONCLUSION: The new boron DDS with A6K/BSH complex is prospective drug for next generation of BNCT.

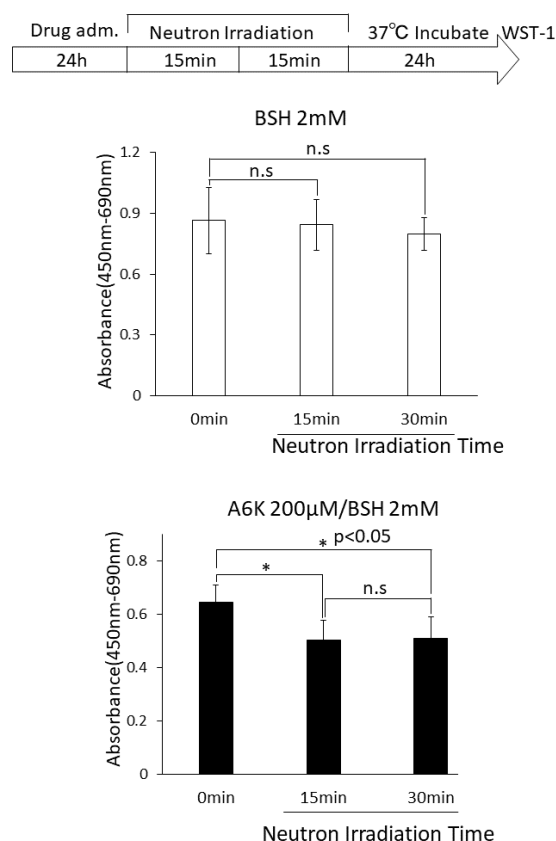


Fig.1 WST-1 assay results after administration of BSH or A6K/BSH complex and irradiation of neutron 0, 15min or 30min.

Functionalization of Hexagonal Boron Nitride Nanosheet with Polyglycerol and its Biomedical Application

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We have reported that polyglycerol (PG) functionalization gives better hydrophilicity than polyethylene glycol (PEG) and is applicable to a wide variety of nanoparticles such as nanodiamond [1] and iron oxide nanoparticle [2]. In addition, a number of hydroxy groups in PG can be used for further derivatization to add more functions to the nanomaterials as a drug carrier and an imaging probe [3]. In this paper, hexagonal boron nitride (h-BN) is functionalized with PG followed by indocyanine green (ICG) derivative to give the imaging probe with near infrared (NIR) fluorescence and good aqueous dispersibility.

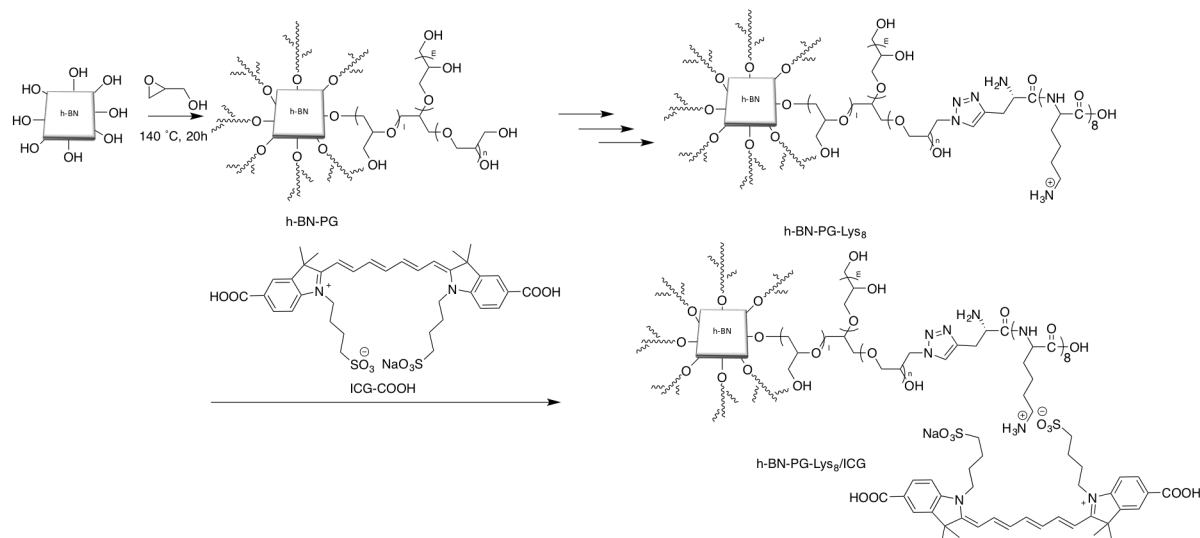
h-BN was functionalized with PG through ring opening polymerization of glycidol as shown in Scheme 1. We confirmed PG functionalization with FT-IR, and solution phase ^1H and ^{13}C NMR. The resulting h-BN-PG exhibited good aqueous dispersibility (2 mg/mL) due to the relatively high PG weight ratio of 25%. Further derivatization of h-BN-PG was carried out to immobilize the ICG derivative as

shown in Scheme 1. The hydroxy groups were converted to azido ($-\text{N}_3$) through tosylate ($-\text{OTs}$), and the resulting h-BN-PG- N_3 was conjugated with octalysine (Lys_8) by click chemistry. Indocyanine green (ICG) derivative was immobilized through electrostatic interaction between Lys_8 and ICG derivative [4]. The obtained h-BN-PG- Lys_8/ICG will be applied to in vitro and in vivo cancer imaging.

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Scheme 1. Synthesis of h-BN-PG- Lys_8/ICG from h-BN through h-BN-PG and h-BN-PG- Lys_8 .



PR10-4 Exploring the Use of the Chicken Egg CAM Assay as an Animal Model for BNCT

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INTRODUCTION: Tamanoi lab has recently established the chicken egg CAM model as an attractive model for studying therapy options for ovarian cancer [1]. In this model, ovarian cancer cells are transplanted onto the CAM (chorioallantoic membrane) of fertilized eggs. Tumor is formed within three to four days. The aim of our experiment is to investigate whether this model can be used as an animal model to examine efficacy of BNCT therapy.

EXPERIMENTS: Fertilized chicken eggs were incubated for ten days and then a window was made on the egg shell and OVCAR8 ovarian cancer cells were transplanted onto the CAM membrane. Three days later, tumor was formed. After injecting ¹⁰BPA into the blood vein that runs through the CAM membrane and is connected to the chick embryo (2.35 mg BPA per egg), the eggs were exposed to neutron at the nuclear reactor for 1 hour. Effect of the tumor was examined by observing tumor size as well as by examining tumor weight two days after the exposure.

RESULTS: As shown in Fig. 1, relatively large tumor was formed three days after ovarian cancer cell transplantation. The tumor was observed easily before the neutron exposure (Day 0). However, two days after the exposure, the size of the tumor observed was much smaller (bottom panel).

The upper figure shows the measurement of tumor weight. The weight of tumor was significantly smaller when the eggs were injected with BPA and then exposed to the neutron beam. Compared with this combined treatment, tumor weight was higher with a single treatment either with BPA (BPA only) or with neutron exposure (neutron only).

Five eggs were used per group in these experiments. One egg was used to measure the amount of boron in the tumor. This was measured to be 12.1 ppm.

In this experiment, we had to examine tumor two days after the neutron exposure due to scheduling constraint. Ideally, the tumor weight should be measured at three or four days after the neutron exposure.

CONCLUSION AND FUTURE PROSPECTS:

Our results suggest that the chicken egg CAM model can be used to examine the effect of BNCT therapy. Instead of BPA compound, other types of compounds carrying boron-10 can be used. In the next experiment, we plan to use tumor targeting nanoparticles containing BPA.

The chicken egg CAM model has a number of attractive features as an animal model. First, tumor can form rapidly in this model. Second, large number of eggs could be used for experiments. Finally, eggs are inexpensive, as the cost of an egg is less than 100 yen.

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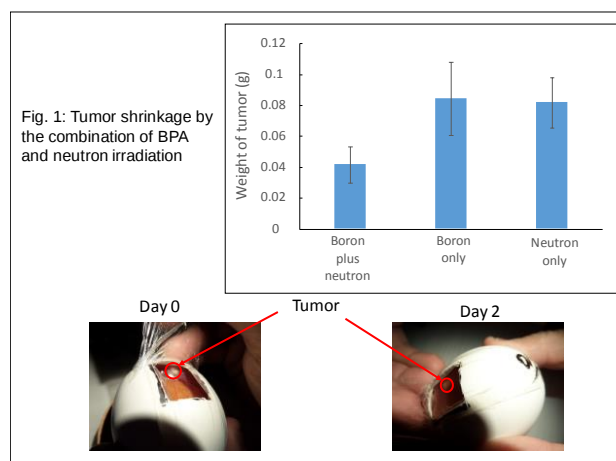


Fig.1. Tumor size and weight after BPA injection and neutron exposure.

PR10-5 Next Generation A (Aomori) - Research and Development of Novel Boron Drugs in BNCT Therapy

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INTRODUCTION: In Aomori Prefecture, "Prefectural Government Refuge against the shortest life time with high death rate" is taken as the prefectural government, Hirosaki University (Faculty of Medicine / Graduate School of Science and Engineering) received this, "Next generation A (Aomori) - Research and development on BNCT treatment "started. The main core technologies related to next-generation A-BNCT therapy are (1) development of new boron medicines, (2) A-BNCT treatment technology and (3) POST / BNCT regeneration technology, and this joint research aims at technological development for practical application concerning (1). New boron drug development In Hirosaki University, IF7-B series (10B-IF7) in which boron nuclide 10B is bound to 7-mer peptide (IFLLWQR; IF7 peptide) 1) -2) which selectively binds to tumor vascular endothelial cells , 10 BSH-IF 7, 10 BPA-IF 7). In this core research, demonstration experiments on the pharmacokinetics, toxicity test and BNCT treatment effect for practical application of the relevant IF7-B series are conducted by nuclear reactor irradiated animal experiments

EXPERIMENTS: In the fiscal year ending March 31, 2010, we will mainly focus on pharmacokinetic examinations by small animal experiments of IF7-B series drugs (3 species). An animal tumor model in which mouse bladder cancer cells (MBT 2) were seeded at the mouse thigh in Hirosaki University was prepared and injected with a boronic agent (IF 7 - BPA, IF 7 - BSH, BPA, BSH) from the mouse tail vein. Mice were sacrificed after drug administration (after 20 minutes, 40 minutes, 60 minutes, 90 minutes, 120 minutes, 240 minutes), and the tumor area and normal organs (liver, kidney, heart, bladder, lung , Brain, spleen, skin, blood) to Teflon containers (10 in total) and then transported to the Kyoto University Reactor. After transportation, measured boron concentration in prompt gamma ray assay(PGA) in E3 duct compartment.

RESULTS: As shown in Fig. 1, sodium borocaptate (BSH). Fig. 1 shows the ¹⁰B concentration in the organ of 13 sites taken out from the mouse specimen after 20 minutes of BHS (Fig.1 (a)) and the new boron drug IF 7-BSH (Fig.1 (b)) by tail injection, respectively. However, in experiments with IF7-BSH, experiments were conducted using multiple specimens. Immediately after injection of both boron drugs (after 5 minutes), the concentration of 10B in various organs including the tumor tissue once increased, but showed a tendency to decrease with the lapse of time. In contrast, in the tumor tissue 10B concentration accumulation ad-

vanced with time and increased to about 35 ppm at the lapse of 20 minutes after injection. As a result, when comparing the accumulation of both boron drugs, the accumulation of IF7-BSH was higher than that of BSH.

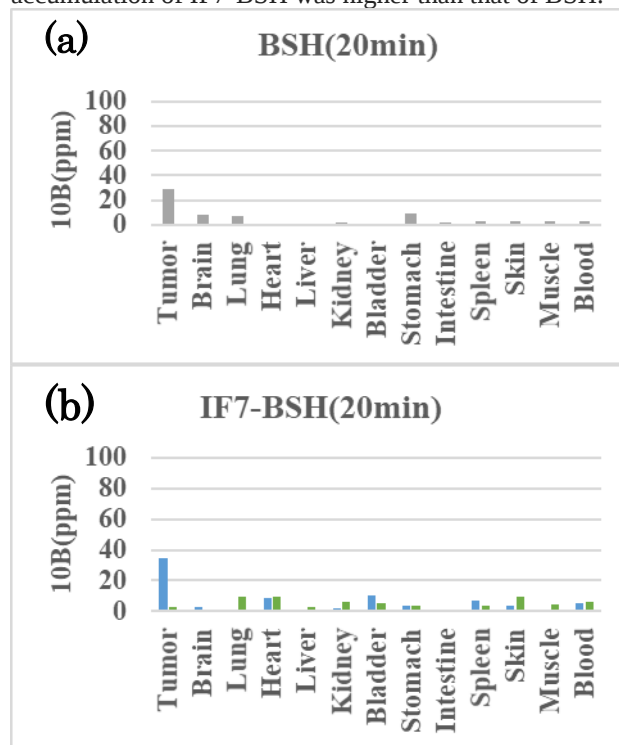


Fig. 1. ¹⁰B condensation in various internal organs including tumor site. In the case of BSH(a) and IF7-BSH. In comparison with BSH, a low level of accumulation was achieved in tumor tissue at 20 minutes after administration of IF7-BSH.

In conventional BSH, since it is not incorporated into the tumor cell, too high accumulation degree can not be obtained at the tumor site. On the other hand, IF7 constituting IF7 - BSH was designed to target ANNEXIN1 which precipitates outside the vascular endothelial cells when tumor tissue forms new blood vessels. Therefore, in this animal experiment it was thought that high ¹⁰B enrichment in tumor tissue could be achieved mainly because IF7 of this compound played the role of boron carrier.

In comparison with BSH, a low level of accumulation was achieved in tumor tissue at IF 7 20 minutes after administration.

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PR10-6 Spherical Particle Fabrication of Boron-Iron Complex Material for BNCT Agent with Magnetic Property

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INTRODUCTION: Submicrometer-sized spherical particles of boron compounds are expected to show high therapeutic efficiency in BNCT due to the high B content of the particle. For example, boron carbide (B_4C) particle with 200 nm in diameter contains 4.6×10^8 of B atoms. Our group attempted submicrometer-sized spherical particle fabrication of boron-iron complex for BNCT agent with an enhanced MRI contrast. In this study, a crystalline structure and chemical composition of fabricated particles composed of boron and iron were analyzed.

EXPERIMENT: Mixture of amorphous boron particles and $\alpha\text{-Fe}_2\text{O}_3$ particles suspended in ethanol was stirred with a propeller driven by a rotational device. The suspension mixture was irradiated with a Nd:YAG laser (pulse width: 7 ns, wavelength: 532 nm, pulse frequency: 30 Hz) for 24 h. Submicrometer-sized spherical particles were obtained by this laser irradiation [1]. The suspended particles after laser irradiation were collected with a centrifugation. The collected particles were treated with 1 M HCl aqueous solution to selectively remove unreacted $\alpha\text{-Fe}_2\text{O}_3$ particles for 6 h, followed by a magnetic separation using a neodymium magnet to eliminate boron and/or boron carbide particles without iron inclusion. Obtained particles were analyzed using an X-ray diffraction (XRD), a high-angle annular dark field scanning transmission electron microscope (HAADF-STEM), and an X-ray photoelectron spectroscopy (XPS).

RESULTS: XRD pattern of obtained particles is shown in Figure 1. The particles contained FeB and B_4C crystalline phases. Figure 2 depicts HAADF-STEM image and elemental distribution images of B, Fe, C, and O. By comparing these images, most particles contained both B and Fe. This result indicates that most particles formed complex of FeB and B_4C and/or B, and is consistent with the XRD result. However, these particles also included O. Therefore, these magnetically collected particles consisted of crystalline FeB, and B_4C , and amorphous compound of Fe and O and/or Fe, B, and O. According to XPS analysis, the atomic ratio of B, Fe, and O in the obtained particles was 5, 50, and 45 %.

A fabrication of Fe-B complex particles with high B content is still a future challenge.

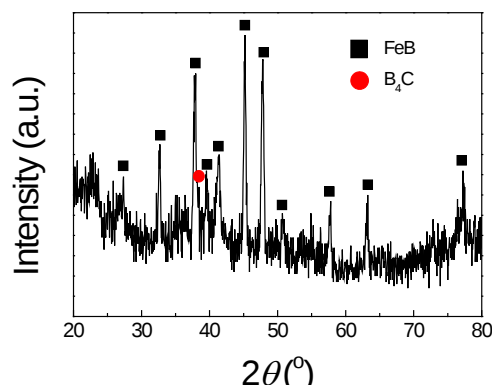


Fig. 1 XRD pattern of magnetically collected particles.

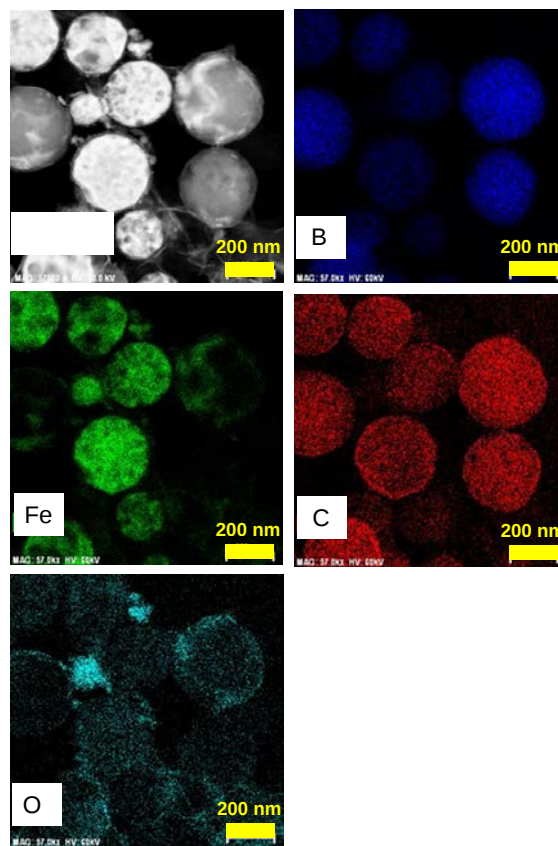


Fig. 2 HAADF and elemental distribution images of magnetically collected particles.

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PR10-7 **Gadolinium-loaded Chitosan Nanoparticles for Neutron Capture Therapy of Cancer: Influence of Particle Size on Tumor-killing Effect *in vitro***

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INTRODUCTION: Gadolinium neutron capture therapy (Gd-NCT) is cancer therapy that utilizes γ -rays and electrons emitted as a result of $^{157}\text{Gd} (n, \gamma) ^{158}\text{Gd}$ reactions. We have been developing gadolinium-loaded chitosan nanoparticles (Gd-nanoCPs) as a means of controlling Gd delivery in Gd-NCT. Accumulation of Gd in Gd-nanoCP-treated tumors is based primarily on the bioadhesive (cationic), biocompatible (nontoxic), and biodegradable (bioerodible) properties of chitosan nanoparticles. Our previous studies demonstrated that neutron-capture reactions after intratumoral (i.t.) injection of Gd-nanoCPs in tumor-bearing mice can significantly suppress tumor growth; however, the inhomogeneous distribution of Gd-nanoCPs in tumor masses prevents complete cure [1]. In addition, it is not clear how γ -rays and electrons relate to the tumor-killing effect. One can expect that reducing chitosan particle size in Gd-nanoCPs will improve the heterogeneous distribution of Gd in tumor tissues and increase the tumor-killing effect of electrons by shortening the adhesion length between Gd-nanoCPs and tumor cells. Thus, we aimed to investigate the effect of nanoparticle size on the tumor-killing effect of Gd-nanoCPs in Gd-NCT.

EXPERIMENTS: Gd-nanoCPs were prepared with chitosan and Gd diethylenetriamine pentaacetic acid (Gd-DTPA) using a water-in-oil (w/o) emulsion-droplet coalescence technique [2]. Two grades of chitosan with different molecular weights (MWs; 10 and 950 kDa) were used to manipulate Gd-nanoCP particle size. B16F10 mouse melanoma cells were employed to evaluate the cellular association properties of Gd-nanoCPs and the tumor-killing effect of thermal neutron irradiation. Tumor-killing effect was evaluated by a cellular viability assay after thermal neutron irradiation.

RESULTS: The use of two grades of chitosan made it possible to obtain Gd-nanoCPs of different sizes and Gd content: Gd-nanoCPs prepared using chitosan with a higher MW (950 kDa) had a mean particle size and Gd content of 468 nm and 7.5 wt%, respectively

(Gd-nanoCP-400); Gd-nanoCPs prepared using chitosan with a lower MW (10 kDa) had a mean particle size and Gd content of 185 nm and 24 wt%, respectively (Gd-nanoCP-200). The tumor-killing effect of Gd-nanoCPs in the Gd-NCT groups was significant, but efficacy was dependent on the micrometric properties of Gd-nanoCPs. Most notably, Gd-nanoCP-200 exhibited a stronger tumor-killing effect than did Gd-nanoCP-400 at the same Gd dose, and the tumor-killing effect of Gd-nanoCP-200 was the same as that of Gd-nanoCP-400 at less than half the Gd-nanoCP-400 Gd dose. This tumor-killing effect could be ascribed to the higher association between Gd-nanoCPs and tumor cells; improved distribution of Gd in cells exposed to Gd-nanoCP-200; and increased influences due to Auger and Coster-Kronig electrons, which have shorter path lengths and stronger tumor-killing ability than do γ -rays. Indeed, cells associated with uptake and adhesion that were exposed to Gd-nanoCP-200 had significantly higher Gd concentrations than those exposed to Gd-nanoCP-400 at less than half the Gd-nanoCP-400 Gd dose. Then the Gd concentration reached $38 \mu\text{g} / 10^6$ cells for Gd-nanoCP-200 at $15 \mu\text{g} \text{ Gd/mL}$ culture medium and $17 \mu\text{g} / 10^6$ cells for Gd-nanoCP-400 at $40 \mu\text{g} \text{ Gd/mL}$ culture medium, respectively. Our results demonstrated that reducing Gd-nanoCP particle size is an effective way to improve cellular affinity for Gd-nanoCPs and enhance the tumor-killing effect of Gd-NCT.

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1st study

Introduction

Folic acid (FA) has high affinity for the folate receptor (FR), which is limited-expressed in normal human tissues and over-expressed in many tumor cells, including glioblastoma [1,2]. We developed a novel pteroyl closo-dodecaborate conjugate (PBC) in which the pteroyl group is known to interact with FR. The purpose of this study was to evaluate the therapeutic efficiency of PBC using F98 glioma-bearing rats *in vivo* boron neutron capture therapy (BNCT).

Materials and Methods

We used two boron compounds; Boronophenylalanine (BPA) and PBC. For *in vivo* study, F98 glioma bearing rats were divided to five groups: untreated controls, neutron irradiation controls, BNCT with BPA (i.v.), BNCT with PBC (CED), and BNCT with combination of BPA (i.v.) and PBC (CED).

Results

Median survival times (MST) of untreated and irradiated controls were 23 and 26 days, respectively, while rats that received PBC(CED), followed by BNCT, had a MST of 31 days, which were similar to those obtained following i.v. administration of BPA (30 days). And the combination group had a MST of 38 days. In combination of PBC (CED) and BPA (i.v.), a significant prolongation in survival time was obtained compared with the single agent groups.

2nd study

Introduction

BPA used in BNCT is a ¹⁰B-derivative of phenylalanine transported into tumor cells by neutral amino acid transporters. 5-aminolevulinic acid (ALA) is also a natural amino acid selectively accumulating in neoplastic cells and inducing photoactivatable porphyrins, mainly protoporphyrin-IX (PpIX) [3,4].

Materials and Methods

In the present study, we examined whether ALA can sensitize glioma to BPA-based BNCT.

We used two cell line; a human glioma stem cell (GSC) line; GB13 and a mouse GSC line; TS. For *in vivo* BNCT, GB13-intracerebrally implanted animals were divided into 6 groups; control, ALA-only, neutron-irradiation only, ALA-neutron irradiation, BNCT only, ALA-BNCT. 80 mg/kg of ALA was orally given to animals 24 hours prior to BPA administration.

Results

The ALA-BNCT group (28 days) obtained a significant prolongation in survival time compared with BNCT only group (25 days).

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PR10-9 Development of S-Alkylthiododecaborate Containing Amino Acids for BNCT

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INTRODUCTION: Boron-neutron capture therapy (BNCT) is based on the nuclear fission reaction of a ¹⁰B-atom with thermal and/or epithermal neutrons to yield high linear energy transfer α particles (⁴He) and recoiling ⁷Li nuclei in tumor cells, and has attracted attention in terms of its potential therapeutic effects on malignant brain tumors, head and neck cancer, and melanoma [1]. A boron delivery agent (boron carrier) with high therapeutic efficiency and low adverse effects is crucial for successful BNCT. For a boron compound to be successful in BNCT, the following criteria must be met: high tumor-targeting selectivity (T/N > 3–4:1), low systemic toxicity, and boron concentration of 20 μ g ¹⁰B/g in tumor tissues. Although many kinds of boron compounds such as amino acids, nucleic acids, and sugars have been reported as boron carriers for BNCT, only two compounds are used clinically for the treatment of cancer using BNCT: *p*-borono-L-phenylalanine (BPA) and mercapto-*c*-closo-undeca-hydrododecaborate (BSH) (Fig. 1).

While developing a new boron carrier for BNCT, we have designed and synthesized a thiododecaborate ([B₁₂H₁₁S]²⁻) unit containing L-amino acids. In vitro evaluation of a BSH-amino acid (DBA, **1**) suggested that DBA might be a potential delivery agent for BNCT [2]. To develop a new boron carrier for BNCT, we designed and synthesized a novel thiododecaborate unit containing amino acids (AS-DBA, **2**), in which the B₁₂H₁₂ cluster is linked to the organic moiety through alkylated S⁺ λ^3 sulfonyl groups. These novel boron compounds have enhanced hydrophobicity and cell membrane permeability owing to a reduced negative charge (–1) and the introduced alkyl chain. Here, we present the biological evaluation of novel boron compounds **2a–g** as boron carriers for BNCT.

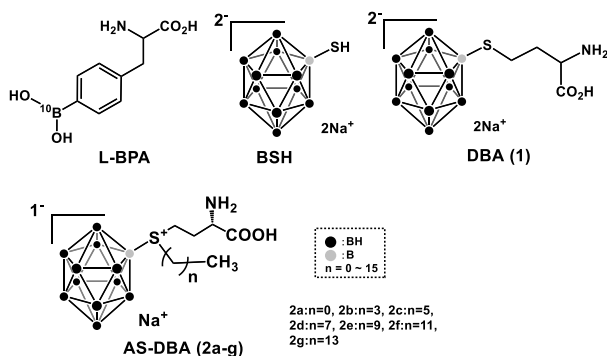


Fig. 1. Boron compounds for BNCT.

RESULTS and Discussion: To evaluate the AS-DBAs, we examined the cytotoxicity, water solubility, and cellular uptake of AS-DBAs and compared them with that of BPA, BSH, and DBA.

The water solubility of **2a–g** was higher than that of BPA (BSH, **1**, **2a–g**: >40 g/L, BPA: 1.6 g/L). The cytotoxicity of AS-DBA **2a–g** was marginally low (IC_{50} >0.1 mM in C6 glioma cells). However, the cytotoxicity of AS-DBAs was higher than that of L-BPA (IC_{50} >10 mM).

In the next step, we measured the boron concentrations in C6 glioma cells by ICP-OES (Fig. 2). The AS-DBAs with a short alkyl chain (**2a–c**, n = 0–5) delivered a small amount of ¹⁰B atoms to C6 cells, whereas AS-DBAs with medium alkyl chains delivered a large amount of ¹⁰B atoms (**2d–g**, n = 0–5). In particular, the intracellular boron concentration of dodecylated AS-DBA **2f** in C6 cells was five times greater than that of L-BPA, with fewer doses of the drug (**2f**: 0.1 mM, BPA: 1.2 mM).

Our results show that AS-DBAs with medium alkyl chains **2d–2g** are useful as ¹⁰B carriers. In vivo evaluation of AS-DBAs are ongoing and the results will be reported soon.

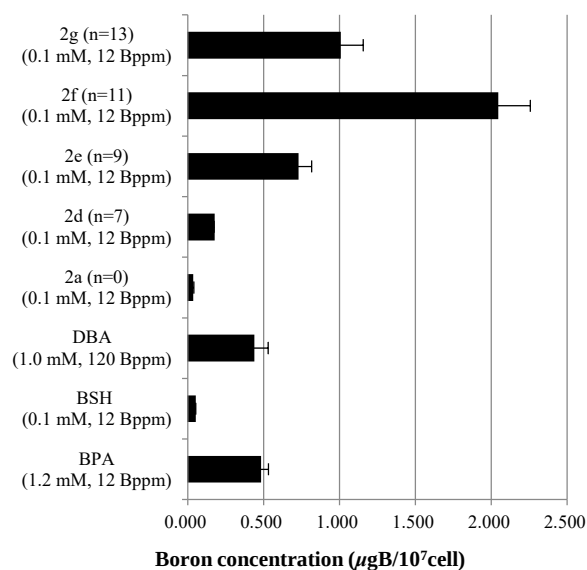


Fig. 2. Amount of boron compounds incorporated into C6 glioma cells.

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INTRODUCTION

Boron neutron capture therapy (BNCT) is gaining attention as a state-of-the-art minimally invasive cancer treatment [1]. Up to now, clinical studies using boronophenylalanine (BPA) and sodium borocaptate (BSH) as ¹⁰B delivery agents for neutron capture reaction have been conducted [2,3]. However, continuous administration of their high concentrations is needed to keep sufficient ¹⁰B tumor concentration. Therefore, we have developed novel boron-containing low molecular compounds efficient in accumulation and retention in tumor.

MATERIALS AND METHODS

3x10⁶ of mouse colon carcinoma (CT26) cells were injected in the right thigh of 5-week-old female Balb/c mice. Two weeks after injection tumor-bearing (avg. 324 mm³) mice were grouped as follows: BN1229, BN1242, BSH, radiation only, and without treatment. BN1229 (57 mg[¹⁰B]/kg, n=8) and BN1242 (57 mg[¹⁰B]/kg, n=8) were injected 24 hours before irradiation. BSH (100 mg/kg with 57 mg[¹⁰B]/kg, n=5) was injected 2 hours before irradiation for comparison. Groups with radiation only (n=7) and without treatment (n=6) were used as controls.

The irradiation was performed with thermal neutrons with a flux of 1.6-3.5 x 10¹² neutrons/cm² over 1 hour.

The tumor size (mm³) was calculated as the long diameter multiplied by the short diameter squared and further divided by 2, in the period starting prior the treatment till 26 days after irradiation.

RESULTS

BN1229, BN1242, and BSH groups showed decrease in tumor size compared to controls (Fig.1 a). The tumor size was independent of the body weight in all mice (Fig.1 b). Because of the large variation in tumor size at the time of grouping, comparison between groups on the 26th day after irradiation was done in mice with a tumor size of 200 mm³ or less before irradiation. A significant tumor growth inhibitory effect was observed in BN1242 group compared

to the untreated control 26 days after irradiation (Fig.1 c-1). The BN1242 compound also showed tendency of efficiency in tumor size decrease over BSH and irradiation only. We assume that BN1242 is a candidate boron compound for further investigation that showed high accumulation in tumor even 24 hours after injection.

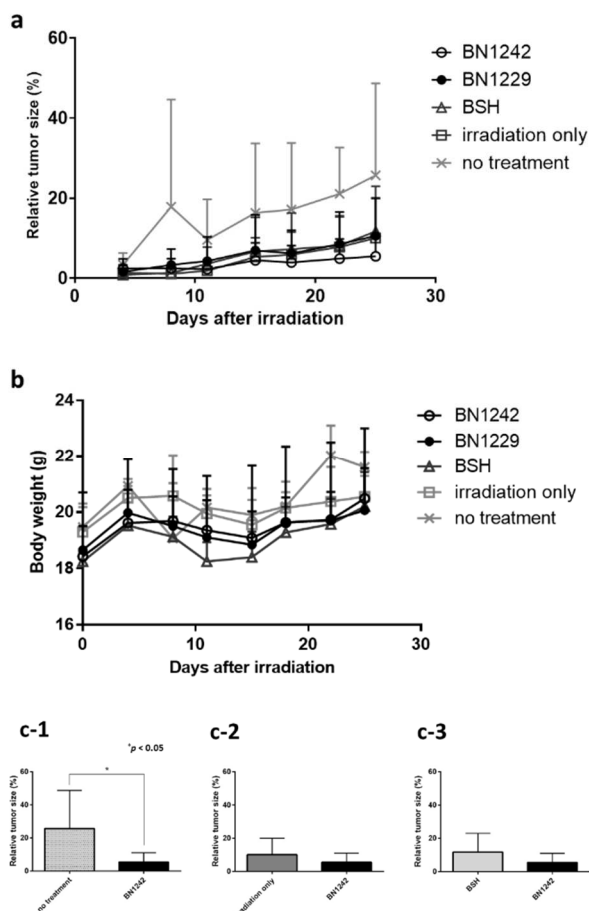


Fig. 1. BNCT of tumor-bearing mice with ¹⁰B-enriched compounds. (a) Tumor growth ratio after 1 hour - thermal neutron irradiation (1.6-3.5 x 10¹² neutrons/cm²) with the injection of BN1242 or BN1229 24 hours, and BSH 2 hours before irradiation, and irradiation only and untreated groups as controls. (b) Mice body weight after thermal neutron irradiation. (c) Tumor growth comparison in mice with initial tumor size of 200 mm³ or less between BN1242 (n = 7) and (c-1) untreated control (n = 3), (c-2) irradiation only (n = 4), and (c-3) BSH (n=4) 26 days after irradiation.

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PR10-11 Development of an Actively-Targeted, Phenylboronic Acid-Installed Nanoparticle Towards Next-Generation Boron Neutron Capture Therapy

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INTRODUCTION

The boron neutron capture therapy (BNCT) is based on binary approach that combination of non-invasive thermal neutron irradiation and administration of boron-10 compounds can result in small-range nuclear fission, followed by tumoricidal effects. During the last decade, much efforts have been made for the establishment of safe and stable neutron sources such as linear accelerators, so that in the near future the BNCT may be propagate much rapidly, through the local hospitals.

Nevertheless, most of the BNCT agents, whether in pre-clinical development or clinical trials, have shown significant obstacles, limiting further clinical translations. For instance, one of only clinically approved BNCT agent, boronophenylalanine (BPA), is a low-molecular weight compound with extremely rapid renal clearance, thus requires continuous injection through the vein for few hours, even during the irradiation. The boron concentration in the blood circulation at the moment of neutron irradiation should be high, not only narrowing down the therapeutic window of the BNCT agent, but also inducing critical damage to the healthy tissues.

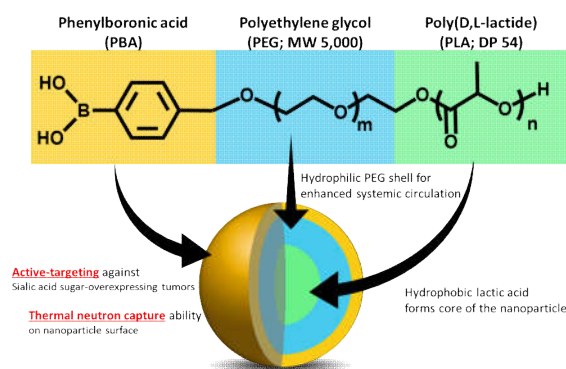


Figure 1. Schematic design of the PBA-installed nanoparticle (PBA-NP)

Herein, to address aforementioned issues by enhancing systemic circulation as well as tumor-specific accumulation of the BNCT agent, our group designed and synthesized a polymeric nanoparticle, as shown in Fig. 1. This nanoparticle is decorated with phenylboronic acid (PBA) groups on the surface, granting thermal neutron capture ability as well as sialic-acid targeting ability. The cellular expression level of sialic acid is known to be linked to the metastatic ability of malignant tumor cells, thus our developed PBA-decorated nanoparticle (PBA-NP) is supposed to be selectively bind on and accumulated in highly metastatic tumor cells. Moreover, being protected by PEG chains, the core-shell supramolecular structure of the PBA-NP is likely to prevent aggregation and degradation in the systemic circulation, may result in extremely high tumor/blood (T/B) ratio after one-shot intravenous injection, by when the PBA-NP is highly accumulated in the tumor tissue, while it is already excreted from the systemic circulation.

EXPERIMENTS

The block copolymer consists of PBA-PEG and PLA (PBA-PEG-*b*-PLA) was synthesized by anionic ring-opening polymerization of ethylene oxide (EO) and D, L-lactic acid on alkoxyated 4-carboxyphenylboronic acid. Then, the PBA-PEG-*b*-PLA was dissolved in dimethylformamide (DMF), followed by dialysis against pure water, to remove organic solvent and to generate the nanoparticles by oil-in-water (o/w) emulsion method. *In vitro* tumor cell recognition ability of the prepare nanoparticles (PBA-NP) was evaluated by confocal laser scanning microscope (CLSM) observation of highly metastatic human breast cancer cells (MDA-MB-231) as well as bovine aortic endothelial cells (BAOEC), pre-treated with PBA-NP or control nanoparticles that having no active PBA groups on the surface. For the evaluation of *in vivo* tumor-targeting ability, orthotopic skin cancer model was prepared by intradermal injection of mouse melanoma cell line (B16-F10) to the C57BL/6j mice, followed by intravenous injection of PBA-NP, sacrificed 48 hr post-injection, harvested the tumor, prepared cryo-sections, then observed with CLSM.

RESULTS

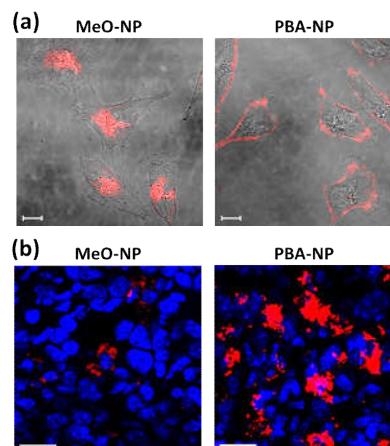


Figure 2. Selective accumulation property of the PBA-NP, compared with methoxy group-installed control nanoparticle (MeO-NP). (a) *in vitro* cancer cell membrane recognition ability of the PBA-NP, MDA-MB-231 cells co-incubated with the nanoparticles for 5 mins. (b) *in vivo* tumor targeting effect of the PBA-NP, observed on cryo-sections prepared from the tumors harvested 48 hr-post intravenous injection. (Blue: Nuclei, Red: Rhodamine-labeled nanoparticles, Scale bars = 20 μ m)

Prepared nanoparticles were stable in physiological salt concentration and 10% serum containing cell culture media (data now shown). As shown in Fig. 2a, PBA-NP could recognize and bind on cellular membrane of sialic-acid overexpressing MDA-MB-231 cells within 5 mins of co-incubation, while the control nanoparticles (MeO-NP) were instantly internalized into the cytosol, presumably by endocytosis. This result strongly suggests that the PBA-NP would be selectively bound on cancer cell membrane, likely to facilitate tumor-specific accumulation, while the MeO-NP would be non-specifically internalized into the normal cells. In accordance with *in vitro* evaluations, *in vivo* intratumoral distribution of the PBA-NP after the intravenous injection, shown in Fig. 2b also supports its selective accumulation ability on *in vivo* administration.

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INTRODUCTION: Boron neutron capture therapy (BNCT) is one of powerful therapies for local tumor control in the treatment of brain tumor, melanoma, and so on [1]. To date, only two boron-containing drugs, L-4-boronophenylalanine (BPA) and BSH (sodium mercaptoundecahydridodecaborate, $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$), have been approved as clinically test compounds, and discovery of better BNCT agents is highly required. Our object in this work is to develop new methods for the tumor-specific accumulation of boron-containing compounds and the real-time detection of B concentrations in local tumor tissues.

Recently, we reported on a new concise and versatile synthesis of the derivatives of sulfoquinovosyl acylpropanediol (SQAP), which has been reported to show a variety of biological activities, including accumulation in tumor cells and the inhibition of tumor cell grow. For instance, a SQAP derivative having o-carborane unit (**4**) was synthesized via substitution reaction of the intermediate **2** and **3** [2]. However, intracellular uptake of **4** in cancer cells was not so high as those of BPA (+ fructose) and BSH.

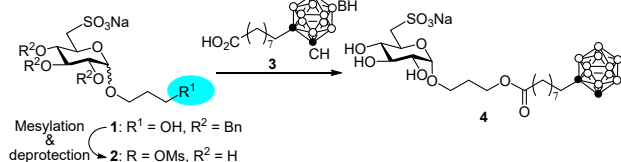


Fig 1. Synthesis of SQAP derivatives bearing o-carborane moiety.

These results prompted us to design and synthesize new B-carriers based on the glucose and glucosamine scaffolds, because it is known that facilitative glucose transporters (GLUTs) and sodium dependent glucose transporter (SLGTs) are highly expressed on cancer cells. For instance, GLUT1 is broadly overexpressed in various cancers, including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian, and cervical. Therefore, glucose and glucosamine analogs that have Michael

acceptor units to accommodate thiols were designed and synthesized in this work.

EXPERIMENTS and RESULTS:

Design and synthesis of new carriers of boron-containing compounds.

In this work, we designed and synthesized a D-glucosamine derivative having a maleimide moiety, which functions as a conjugate acceptor of various thiols such as BSH, at the 2-position (**5** in Fig. 2), of D-glucosamine, which was easily available from D-glucosamine, as shown in Fig. 2. The synthesized **5** was reacted with BSH and other thiol derivatives having boron to obtain **6**. Cytotoxicity of the analogs of **6** to cancer cells such as HeLa and A549 cells was evaluated by MTT assay and their intracellular uptake to these cancer cells was examined by ICP-MS (inductivity coupled plasma-mass spectrometer). It was found that intracellular uptake of these compounds are lower than our reference compound [3], boronobenzyl-cyclen (cyclen = 1,4,7,10-tetraazacyclododecane) [4], albeit these toxicity were relatively low. The improvement of the design of these B-carriers are now in progress.

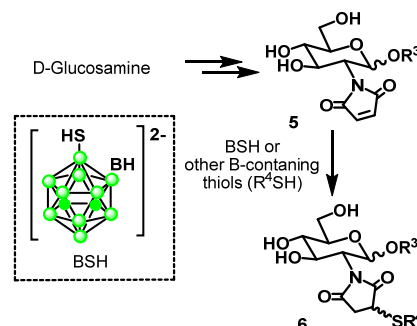


Fig 2. Synthesis of boron carriers based on glucosamine analogs.

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INTRODUCTION: Boronophenylalanine (BPA) is one of the most promising boron drugs for boron neutron capture therapy (BNCT), because it can be taken up selectively by cancer cells through the large neutral amino acid transporter 1 (LAT1), which is reported to be overexpressed on many malignant tumor cells [1]. Although BPA has exhibited efficient accumulation within numerous types of tumors, its therapeutic efficacy has been some-times compromised by untoward quick clearance from the target tumor. The clearance from the tumor cells may be due to antiport mechanism of LAT1; intracellular BPA should be excreted when extracellular BPA concentration is lowered [2].

To facilitate the intracellular retention of BPA, we have recently synthesized finely-tuned poly(vinyl alcohol) (PVA), and prepared the complex of BPA and PVA (PVA-BPA) via the formation of boronic acid-diol bonds. Since PVA-BPA has many BPA molecules in its structure and ex-pose the structure of phenylalanine of BPA, which is re-ported to be the critical structure for interaction with LAT1 [3], PVA-BPA can induce multivalent interaction with LAT1 on tumor cells, resulting in its efficient intracellular internalization through LAT1-mediated endocytosis. Prob-ably because PVA-BPA can be localized in endo-/lyso-somes and avoid untoward efflux from the target cells, PVA-BPA importantly revealed prolonged intracellular re-tention in *in vitro* condition. In addition, in *in vivo* study, PVA-BPA showed efficient tumor accumulation and dras-tically improved tumor retention compared to conven-tional BPA. Thus, in this study, we have examined the therapeutic potential of PVA-BPA in BNCT using Kyoto Uni-versity Research Reactor (KUR).

EXPERIMENTS: BALB/c mice bearing subcutaneous CT26 tumors were used in this study. PVA-BPA or the fructose-BPA complex was intravenously injected to the mouse (10 mg BPA/mouse), and the thermal and epither-mal neutrons were irradiated to the tumor using KUR 3 or 6 h after the injection. Size of tumor was measured using a caliper, and tumor volume (*V*) was calculated using the following equation:

$$V = 1/2 \times a \times b^2,$$

where *a* and *b* denote major and minor axes of a tumor, respectively.

RESULTS: As shown in Fig. 1, fructose-BPA significantly suppressed the tumor growth, indicating its excel-lent clinical therapeutic potential. However, apparent tu-

mor regrowth was observed at day 15, which may be explained by the untoward efflux of BPA from the tumor and the eventually compromised therapeutic efficacy.

In contrast, PVA-BPA exhibited drastic antitumor activity, and tumor regrowth was not observed even at day 18. Since PVA-BPA can efficiently accumulate within the tumor and retain for a prolonged period, PVA-BPA might maintain high intratumoral boron concentration that is sufficient to obtain strong antitumor activity during neutron irradiation (50 min). This explanation is supported by the strong antitumor activity obtained by PVA-BPA and neutron irradiation 6 h after the injection; PVA-BPA could exhibit high intratumoral boron concentration even 6 h after the injection. It should be also noted that histological analysis of the tumor treated with PVA-BPA with 3 h interval between injection and irradiation revealed the cell death of almost all the tumor cells. These results indicate that PVA-BPA may be a promising boron delivery system to accomplish efficient BNCT.

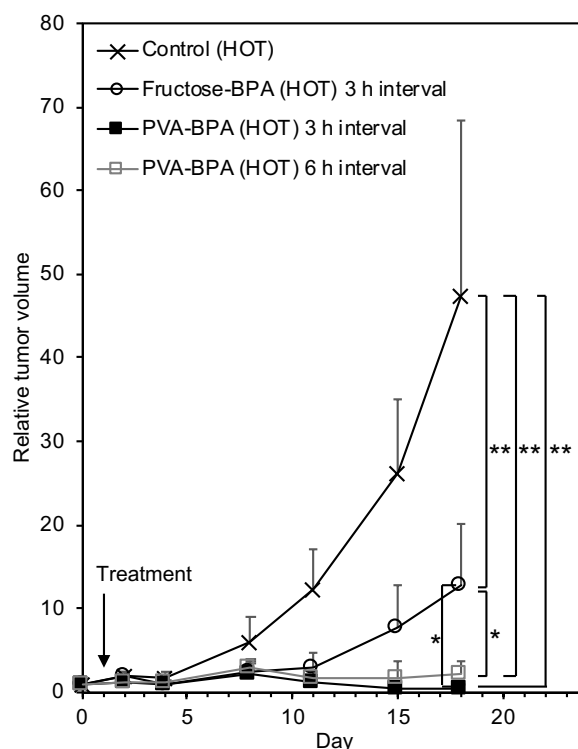


Fig. 1. Antitumor effect of fructose-BPA and PVA-BPA. At day 1, the samples were injected and neutrons were irradiated to the tumor.

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PR10-14 Development of Boron Cluster Containing Water-Soluble Folate Derivatives As Novel Small Molecular Agents for BNCT

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INTRODUCTION: Development of new boron compounds is still indispensable for expanding the application of boron neutron capture therapy (BNCT) for the treatment of various cancers. For successful treatment of cancer by BNCT, a boron compound requires high tumor accumulation, low toxicity and water solubility. In order to achieve these criteria, we focused on folate receptor (FR). FR is a cell surface protein that takes folic acid into the cell by receptor-mediated endocytosis. It is well known that one of the FR, FR α , is overexpressed on the surface of many cancer cells, for example ovary, lung, breast and kidney cancers. Kettenbach et al. developed a carborane-containing folate derivatives that was found to accumulate to KB cells [1], although the water solubility was relatively low. Herein, we designed and synthesized pteroyl *closo*-dodecaborate conjugates (PBCs).

EXPERIMENTS:

U-87MG human brain tumor cells were transferred to the atomic reactor (KUR; Kyoto University Research Reactor) and incubated with the culture medium (RPMI1640) in 96 well plate at a density of 3.0×10^4 cells/well for 12 h. The cells were exposed by L-BPA, PBC1 and PBC4 dissolved in medium at 5, 10, 25 ppmB for 3 h then the cells were irradiated at KUR with reactor thermal neutron beams for 0, 10, 25, 60 min. After neutron irradiation, medium was removed and 100 μ L medium was added. The cells were cultured at 37°C for 22 h, then added 10 μ L MTT (5 mg/mL dissolved in water) and incubated at 37°C for 2 h. After incubation, the medium was removed and 100 μ L DMSO was added. The measurement was performed by reading absorbance 595 nm.

RESULTS: U-87MG cells were incubated with PBC1, PBC4, and L-BPA for 3 h at various concentrations (5-25 ppmB), and neutron irradiation was carried out for 0-60 min. Irradiation time-dependent cell viability was shown in Figure 1. It was expected that BNCT effect higher than that of L-BPA because both PBC1 and PBC4 were highly taken up by U-87MG cells due to highly expression of FR α . Indeed, our preliminary study revealed that both PBC1 and PBC4 were accumulated into U-87MG cells higher than L-BPA. However, the results were not as expected. For example, in the case of PBC1, higher cytotoxicity was observed at doses of 5 and 10 ppmB. The cell viability at 25 ppmB was similar level to that of the

control (no treatment), revealing that neutron flux was not sufficient. In the case of PBC4, highest cytotoxicity was observed at 25 ppmB. L-BPA showed the similar phenomena to PBC1: the highest cytotoxicity was observed at 5 ppmB. Furthermore, the significant cytotoxicity was not observed in all cases: cell viabilities were around 80%. These results indicate that the neutron flux was not sufficient in the current irradiation experiments.

In conclusion, we developed boron cluster containing water-soluble folate derivatives (PBCs) and demonstrated their cytotoxicity with neutron irradiation at KUR. However, significant cytotoxicity was not observed in all cases due to insufficient neutron flux. We will conduct the neutron irradiation again under the modified conditions in the next year.

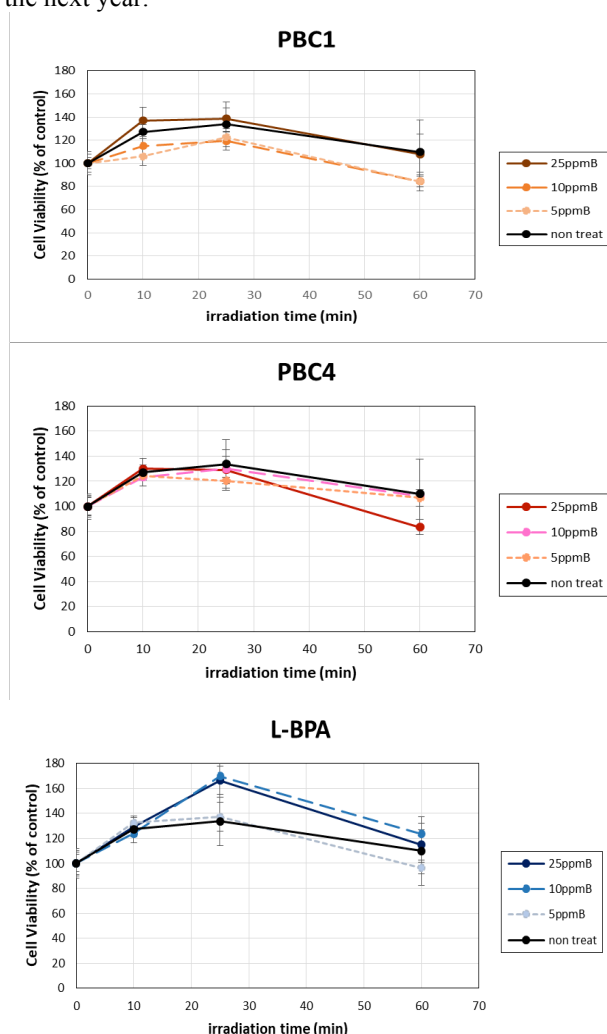


Fig. 1. Survivals of U-87MG cells incubated with various boron compounds after neutron irradiation.

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PR10-15 Evaluation of Novel Boron Liposome *in vivo* by Thermal Neutron Irradiation

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INTRODUCTION: For the tumor treatment, Boron Neutron Capture Therapy (here in after, also referred to as “BNCT”) is attracting attention as one of the radiation therapy. BNCT could be treated only a malignant tumor cells selectively without damage to normal cell compared with conventional radiation therapy.

Recently, as method of boron accumulation, BDS (Boron Delivery System) using DDS (Drug Delivery System) is remarkable in BNCT. A liposome which is widely used as DDS material is prior for BDS.

Therefore, we developed novel lipid (name PBL^[1]) and prepared liposome using this lipid. In this report, we have irradiated the liposome *in vivo*.

EXPERIMENTS: The liposome modified with PBL prepared using the lipids by conventional lipid-film method^[2] and the constant ratio(Table.1). BSH was encapsulated to liposome by freeze-thaw method^[3]. The resulting liposomes were extruded with an extruder through a polycarbonate membrane with a 100-nm pore size, yielding the boron liposome.

Table.1) Lipid construction (molar ratio)

Lipid	DSPC	Cholesterol	PBL
PBL-liposome	48	48	4

The cancer-bearing models were prepared by grafting mouse colon cancer cells (CT26, 3×10^6 cells) to the right thighs of BALB/cA mice (female, 5 weeks old, weighing 16-20 g) to have a tumor diameter of 6-8 mm.

After 2 weeks, each samples (Table.2) were injected to the tail vein of the prepared tumor-bearing mice and, after 48 hours, neutron irradiation was conducted with KUR. BSH was injected 2 hours before irradiation as positive control group. The thermal neutron dose was 5.2×10^{12} neutrons/cm² for 2 hours.

Table.2) Samples and the concentration of ¹⁰B.

Sample	PBL-liposome	BSH-encapsulated PBL-liposome	free BSH
¹⁰ B conc. [mgB/kg]	10	15	57
Lipid conc. [mg/mL]	108	108	—

The tumor size was determined over time after the irradiation until Day 26 so as to compare the effect of inhibiting tumor growth with the control group. The tumor sizes were determined according to the following formula.

$$(\text{Long diameter (mm)}) \times (\text{Short diameter (mm)})^2 / 2 = \text{tumor size (mm}^3\text{)}$$

RESULTS: As shown in Figure 1, the PBL-liposome and BSH-encapsulated PBL-liposome significantly inhibited the tumor growth as compared to other control groups onward Days 11. In particular, the tumor did not grow until Day 11.

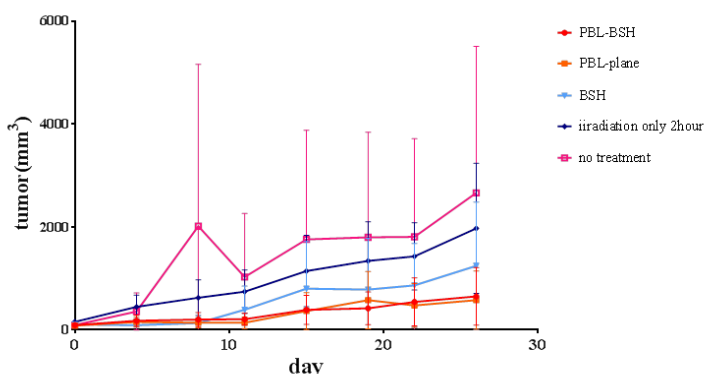


Fig.1) Anti-tumor effect of BNCT by boron liposome.

Tumor growth size after thermal neutron irradiation with the injection of PBL-liposome (PBL-plane, n=3), BSH-encapsulated PBL-liposome (PBL-BSH, n=6) and free BSH (BSH, n=4). As controls, tumor growth size with irradiation only (n=6) and untreated (no treatment, n=3).

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INTRODUCTION: *p*-Boronophenylalanine (¹⁰BPA) and sodium borocaptate (¹⁰BSh) as neutron capture agents show superior selective tumour accumulation property, but low tumour retentivity, so administration several hours before the radiation and persistent administration are necessary for effective BNCT in clinically.

In this study, we synthesized the novel boron compound which had superior tumour retentivity, and evaluated the anti-tumour effect by neutron capture reaction.

EXPERIMENTS: We prepared the tumour bearing mice model after injection of CT26 mouse colorectal cancer cells (3 x 10⁶ cells) into the right femoral region subcutis of the female Balb/c mice.

Two dosage of BN2018 (7.2 mg[¹⁰B]/kg, 14.4 mg[¹⁰B]/kg) as tested compound, and fructose chelate of ¹⁰BPA 300 mg/kg (14.4 mg[¹⁰B]/kg) as a positive control was administrated by tail vein injection under awakening 24 hours (BN2018), 2 hours (¹⁰BPA) before radiation, respectively (each group n=7). As the control group, group of irradiation only (n=9), group of BN2018 (14.4 mg[¹⁰B]/kg) administration(n=4), and untreated group (n=7) were used.

The tumour-bearing mice were irradiated with epi-thermal neutrons of 3x10¹²n/cm² average fluence at the KUR. The reactor power was 1MW, and irradiation was carried out for 60min. After irradiation, the effect of BNCT was evaluated on the basis of tumour volume (calculated as 1/2 x length x width²) until 26th days postirradiation.

RESULTS: The results of epi-thermal neutron irradiation is shown in Fig. 1. The effect on weight in all individuals was not found (a). Tumour growth suppression effect was found in the comparison of the tumor volume increase rate for untreated group in the BN2018 administrated group and the ¹⁰BPA administrated group (b). In comparison between groups of the 26th day post-irradiation in the individual which is with 210mm³ or less of tumour size before the irradiation, the tumor growth suppression effect in the BN2018 (7.2 mg of [¹⁰B]/kg) administrated group is significantly found compared with untreated group (c-1). Only a tendency to suppression was confirmed compared with the group of

irradiation only (c-2). It was also found that the BN2018 had slightly weak depression effect compared to the ¹⁰BPA administrated group (c-3).

We could be confirmed that the tumour growth suppression effects by BNCT in the group of BN2018 (7.2 mg of [¹⁰B]/kg) administrated 24 hours before irradiation is equal to the effects in the group of ¹⁰BPA (14.4 mg of [¹⁰B]/kg) administrated 2 hours before irradiation.

In this study, we showed the tumour growth suppression effects derived the highly tumour retentivity of BN2018 by BNCT. This novel compound has possibility to be applied to BNCT as tumour selective ¹⁰B compound in future.

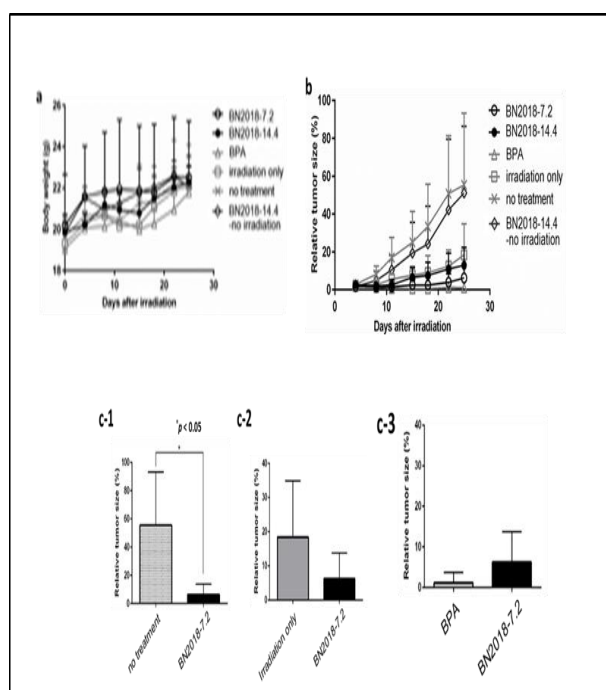


Fig. 1. BNCT for tumor-bearing mice after thermal neutron irradiation for 1 hr (3 x 10¹² neutrons/cm²). (a) Average bodyweight of mice. (b) Tumor growth ratio of tumor-bearing mice with injection of ¹⁰B-enriched BN2018 (7.2 mg[¹⁰B]/kg and 14.4 mg[¹⁰B]/kg) 24 hr before irradiation, ¹⁰BPA (14.4 mg[¹⁰B]/kg) 2 hr before irradiation, or irradiation only. The mice without treatment served as controls. (c) Tumor growth ratio of tumor-bearing mice with a tumor size of 210 mm³ or less before irradiation with injection of ¹⁰B-enriched BN2018 (n = 5, 7.2 mg[¹⁰B]/kg) vs. (c-1) no treatment (n = 5), (c-2) irradiation only (n = 9) and (c-3) ¹⁰B-enriched BPA (n = 7, 7.2 mg[¹⁰B]/kg) 26 days after thermal neutron irradiation.