Title of Paper: Pharmacokinetic study of ^{14}C-radiolabeled *p*-boronophenylalanine (BPA) in sorbitol solution and the treatment outcome of BPA-based boron neutron capture therapy on a tumor-bearing mouse model

Short running title: *p*-boronophenylalanine (BPA) in sorbitol solution for BNCT

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Abbreviation: BNCT, boron neutron capture therapy; BPA, *p*-boronophenylalanine; HPLC, high-performance liquid chromatography; Hct, hematocrit value; tSIE, the transformed spectral index of the external standard; EDTA, ethylenediaminetetraacetic acid; CBE, compound biological effectiveness; RBE, relative biological effectiveness;

Key points

p-boronophenylalanine (BPA) in sorbitol solution maintains stability for longer than that in fructose solution; therefore, it can be stored for a longer period.

Pharmacokinetic studies with ^{14}C-radiolabeled BPA confirmed that the sorbitol solution of BPA is distributed through tumors as BPA in fructose.

Neutron irradiation was found to produce dose-dependent antitumor effects both in vitro and in vivo after the administration of BPA in sorbitol solution.

Abstract:

Background and Objective: Boron neutron capture therapy (BNCT) is a binary cancer treatment that combines boron administration and neutron irradiation. The tumor cells uptake the boron compound and the subsequent neutron irradiation results in a nuclear fission reaction caused by the neutron capture reaction of the boron nuclei. This produces highly cytocidal heavy particles, leading to the destruction of tumor cells. *p*-boronophenylalanine (BPA) is widely used in BNCT but is insoluble in water and requires reducing sugar or sugar alcohol as a dissolvent to create an aqueous solution for administration. The purpose of this study was to investigate the pharmacokinetics of ^{14}C-radiolabeled BPA using sorbitol as a dissolvent, which has not been reported before, and confirm whether neutron irradiation with a sorbitol solution of BPA can produce an antitumor effect of BNCT.

Materials and methods: In this study, we evaluated the sugar alcohol, sorbitol, as a novel dissolution aid and examined the consequent stability of the BPA for long-term storage. U-87 MG and SAS tumor cell lines were used for *in vitro* and *in vivo* experiments. We examined the pharmacokinetics of ^{14}C-radiolabeled BPA in sorbitol solution, administered either intravenously or subcutaneously to a mouse tumor model. Neutron irradiation was performed in conjunction with the administration of BPA in sorbitol solution using the same tumor cell lines both *in vitro* and *in vivo*.

Results: We found that BPA in sorbitol solution maintains stability for longer than that in fructose solution, and can therefore be stored for a longer period. Pharmacokinetic studies with ^{14}C-radiolabeled BPA confirmed that the sorbitol solution of BPA distributed through tumors in much the same way as

BPA in fructose. Neutron irradiation was found to produce dose-dependent antitumor effects, both *in vitro* and *in vivo*, after the administration of BPA in sorbitol solution.

Conclusion: In this report, we demonstrate the efficacy of BPA in sorbitol solution as the boron source in BNCT.

1 Introduction

Boron neutron capture therapy (BNCT) is a binary cancer therapy that utilizes boron-containing drugs and neutron irradiation (Fig. 1a). The nuclei of boron atoms (^{10}B) capture low-energy neutrons at extremely high probability compared with ordinal elements composing tissue. ^{10}B capture low-energy neutrons and fission into alpha particles and lithium nuclei. These are heavy particles with high cytocidal potential. Each particle has a range of less than 10 mm (5–9 mm), less than the diameter of a single tumor cell. Furthermore, the irradiated neutrons are low energy and have fewer biological effects than fast neutron beams or heavy particle beams irradiated externally. Therefore, the selective delivery of boron atoms (^{10}B) to tumor cells using appropriate drug carriers, followed by low-energy neutron irradiation, allows cellular-level radiation therapy (Fig. 1b) [1,2]. This principle behind BNCT was first proposed in 1936, only 4 years after the discovery of neutrons in 1932 [3]. However, the theory took time to come to fruition because therapeutically efficient boron drugs had not yet been developed. An optimal boron drug for BNCT called *p*-boronophenylalanine (BPA) (Fig. 1c) was first proposed in 1967 and applied to patients later in 1987 [4], and both the safety and efficacy of this boron drug were verified, confirming the feasibility of cancer treatment by BNCT [5].

Currently, BPA is widely used in BNCT. It is an amino acid analog with a similar structure to the amino acid, tyrosine. When BPA was first developed, the primary challenge was its extremely poor solubility in water within the physiological pH range. However, the solubility of BPA was found to improve

96 dramatically when it was first dissolved in a monosaccharide solution, making BNCT via intravenous
97 or continuous infusion of BPA possible [6]. Among the monosaccharides, fructose was found to be a
98 relatively suitable agent and many animal and clinical preliminary studies have been conducted using
99 BPA in fructose solution [7]. However, fructose is a reducing sugar that can react with amino compounds
100 to produce melanoidin pigment molecules in a Maillard reaction [8]. As a result, the shelf life of BPA in
101 fructose solution is limited to less than 2 weeks when stored in the dark at 2°C–8ºC [9]. Therefore, an
102 attempt was made to use sugar alcohols (e.g., mannitol, sorbitol) as BPA solubilizers instead of
103 monosaccharides, as sugar alcohols are resistant to heat and pH changes and do not cause the Maillard
104 reaction [10].
105
106 Among sugar alcohols, both sorbitol and mannitol were found to serve as excellent dissolving agents
107 capable of maintaining the long-term stability of BPA. As sorbitol is more soluble in water than mannitol,
108 it is superior to mannitol for use with BPA; thus, BPA in sorbitol is currently used clinically in BNCT.
109 Fructose solution of BPA was prepared by time-conditioning due to its unstable nature. To widely use
110 BPA as a therapeutic drug in clinical practice, it should be distributed to hospitals as a shelf-stable
111 intravenous drip formulation. However, no reports on whether a sorbitol solution of BPA can actually
112 produce therapeutic effects with neutron irradiation for BNCT have been published. Therefore, this
113 study aimed to confirm the antitumor effect of BPA in sorbitol solution on neutron irradiation. To
114 determine the appropriate timing of neutron irradiation of BPA in sorbitol solution, a pharmacokinetic

115 study was conducted using ^{14}C-radiolabeled BPA (Fig. 1d) in sorbitol solution.

116

117 **2 Methods**

118 **2.1 Materials**

119 ^{10}B-enriched BPA, supplied by Stella Pharma Corporation (Osaka, Japan) (purity 99.4% when measured
120 by high-performance liquid chromatography [HPLC]), was used for all experiments in this study. ^{14}C-
121 radiolabeled BPA was purchased from Sekisui Medical Company, Ltd (Tokyo, Japan) (specific
122 radioactivity: 11.6 MBq/mg; purity 98.4% [HPLC]). U-87 MG tumor cell lines were purchased from
123 the American Type Culture Collection (Virginia, USA). SAS human squamous cell carcinoma (tumor
124 of the tongue) cell lines were purchased from Riken Cell Bank (Saitama, Japan).

125

126 **2.2 Bioanalytical assay**

127 For stability tests, LaChrom Elite L-2000 (Hitachi Ltd., Tokyo, Japan) with Atlantis T3 columns (Waters
128 Corp., Massachusetts, USA) was used for HPLC. The measurement conditions were as follows: column
129 temperature, 40°C; HPLC mobile phase water:methanol:trifluoroacetic acid ratio, 950:50:1; flow rate,
130 1.0 mL/min; detection wavelength, UV 223 nm.

131

132 For ^{14}C-radiolabeled BPA experiments, LC-10A (Shimadzu corporation, Kyoto, Japan) with Mightysil
133 RP-18GP columns (S-5 micrometer, 4.6 mm I.D. x 150 mm) (Kanto Reagents, Hokkaido, Japan) was

used for HPLC. The measurement conditions were as follows: column temperature, 40°C; HPLC mobile phase A = 0.05 mol/l sodium dihydrogenphosphate dihydrate (pH 2.5):methanol = 95:5 (v/v) B = 0.05 mol/l sodium dihydrogenphosphate dihydrate (pH 2.5):methanol = 5:95 (v/v); Gradient 0 min (A 100%, B 0%), 25 min (A 62.5%, B 37.5%), 30 min (A 0%, B 100%), 40 min (A 0%, B 100%); flow rate, 1.0 mL/min; detection wavelength, UV 223 nm. Samples were processed using a sample oxidizer (Model 307, PerkinElmer, Inc., Massachusetts, USA) with a glass liquid scintillation vial (No. 986546), carbon dioxide absorbent (Carbo-Sorb 8mL), and scintillation cocktail (Permafluor). For ^{14}C detection, we used FLO-ONE A525 (PerkinElmer, Inc., Massachusetts, USA), Ultima-Flo M (flow rate of scintillation cocktail: 3.0 mL/min). Liquid scintillation counters (Tri-Carb 2500TR and 2700TR, PerkinElmer, MA, USA) were used to measure the radioactivity of each organ with the transformed spectral index of the external standard (tSIE) method. Each sample was measured for 5 minutes. Samples that contained only scintillation cocktails were similarly measured for background data, and the value of the background data was subtracted from the measured value of each tissue sampled and used in the analysis. ADME SUPPORT software (version 2, FUJITSU, Tokyo, Japan) was used to calculate tissue radioactivity concentrations. The detection limit of the radioactivity measurement was set to less than twice the background value.

2.3 BPA preparation and measurement of its stability in sorbitol solution

BPA (3 w/v%) was prepared in an aqueous solution of sorbitol (BPA 300 mg, D-sorbitol 315 mg, total

10 mL) [7,11]. The solution was sterilized with a 0.22-mm filter (Merck Millipore, Massachusetts, USA). The BPA in sorbitol solution (2.5 w/v%) used for stability testing was prepared in the same manner (BPA 250 mg, D-sorbitol 328.1 mg, total 10 mL solution). BPA in fructose solution was prepared in the same way (BPA 250 mg, fructose 555.8 mg, total 10 mL solution). 500 mg/3.87 MBq/16.7 mL ^{14}C-radiolabeled BPA in sorbitol solution was prepared by mixing 0.20 mg ^{14}C-radiolabeled BPA and 299.8 mg BPA. Similarly, 125 mg/3.87 MBq/16.7 mL ^{14}C-radiolabeled BPA in sorbitol solution was prepared by mixing 0.20 mg ^{14}C-radiolabeled BPA and 74.8 mg BPA. Each BPA solution was stored in a chamber at 60 °C (ADP300, Yamato Scientific Co. Ltd., Tokyo, Japan) and changes in appearance, pH, osmotic ratio, and BPA concentrations were assessed after 1, 2, and 4 weeks. Changes in the pH of the solution were measured using a 781 pH/ Ion Meter (Metrohm Japan, Tokyo, Japan). Osmotic pressure to saline ratios were measured using an automatic osmometer (Osmo Station, OM6060, Arkray Inc., Kyoto, Japan). The BPA concentrations were determined using HPLC.

2.4 Tumor cell lines and mouse handling

U-87 MG cells were maintained in the minimum essential medium supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. SAS cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. Both cell lines were cultured at 37 ºC in a 5% CO_2 incubator. The size of the subcutaneous tumor volume of each mouse was

calculated using the formula: 1/2 × length × width × width. The mice used in this study were cared for and treated according to the Recommendations for the Handling of Laboratory Animals for Biomedical Research by the Committee on Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. Additionally, all animal experiments were conducted in accordance with the laboratory animal handling guidelines of our institution.

2.5 Pharmacokinetic experiments using ^{14}C-radiolabeled BPA

5×10^6 U-87 MG cells were injected subcutaneously into the backs of 9-week-old Balb/cA Jcl-nu/nu female mice (CLEA Japan, Tokyo, Japan). Sixteen days after tumor injection, when the U-87 MG tumors reached 200–500 mm^3, the mice were assigned to two groups so the mean tumor volume of each test group was homogeneous (in total, nine mice in each tumor model, three mice at each time point). The mice in one of the groups received intravenous injections of 500 mg/ 3.87 MBq/ 16.7 mL/kg of BPA in sorbitol solution, while the mice in the second group received intravenous injections of 125 mg/ 3.87 MBq/ 16.7 mL/kg body of BPA in sorbitol solution. Injections were via the tail vein. The mice were sacrificed 0.25, 1, and 2 h after injection, and, from each mouse, the blood, subcutaneous tumor, brain (cerebrum and cerebellum), lungs, heart, liver, kidneys, spleen, pancreas, skin on the tumor surface, and muscle adjacent to the tumor were collected. Blood was collected as much as possible from the posterior vena cava by opening the abdomen under inhalation anesthesia. 100 μL of the blood was used to measure the boron concentration in the blood. Half of the blood collected from each mouse was aliquoted into

191 other tube and a hematocrit capillary tube and centrifuged (14,400 × g, 5 min), respectively. A 100-μL
192 aliquot of the supernatant plasma was collected from the former tube and used to measure the boron
193 concentration in the plasma. The latter hematocrit capillary tube was used to measure the hematocrit
194 value (Hct) value.
195
196 Next, we prepared the U-87 MG mice tumor models to examine the pharmacokinetics of 500 mg/ 3.87
197 MBq/ 16.7 mL/kg of BPA in sorbitol solution via subcutaneous administration. Twenty-one days after
198 tumor injection, when the U-87 MG tumors reached 200–500 mm^3, the mice were assigned to each
199 group of time points (1 h, 2 h, 3 h, and 4 h) so that the mean tumor volume among the groups was similar
200 (in total, twelve mice in each tumor model, three mice at each time point). These mice were sacrificed
201 1, 2, 3, and 4 h after treatment, and the blood, brain (cerebrum and cerebellum), lungs, heart, liver,
202 kidneys, spleen, pancreas, small intestines, colon, oral mucosa, skin on the tumor surface, and muscle
203 near the tumor were collected. As the method described above, blood was collected from the posterior
204 vena cava by opening the abdomen under inhalation anesthesia. The distribution of BPA in the normal
205 and tumor tissue of the subcutaneous group of mice was studied in the same way as in the intravenous
206 group. Boron concentrations in the plasma were measured in the same manner as above.
207
208 **2.6 *In vitro* neutron irradiation of tumor cell lines combined with BPA in sorbitol solution**
209 BPA in sorbitol solution was added to the medium to achieve boron concentrations of 10 and 25 ppm (=

μg ^{10}B / g medium). The U-87 MG and SAS cells were separately cultured with BPA for 1 h. The tumor cells were then detached from the flasks with 0.5 g/L trypsin + 0.53 mmol/L ethylenediaminetetraacetic acid (EDTA) solution (Nacalai Tesque Inc., Kyoto, Japan) containing BPA at the same concentration as that in the medium. The cell mixtures were adjusted to cell concentration of 1×10^6 cells/mL, transferred to test tubes, and irradiated with 1.54, 3.08, 4.62, or 6.16×10^{11} n/cm^2 thermal neutron fluence (equivalent absorbed doses: 2, 4, 6, and 8 Gy, respectively). After neutron irradiation, the cells were seeded into dishes for colony-forming assay. Fourteen days after the cells were seeded, the number of colonies was determined and the cell survival rate was calculated.

2.7 *In vivo* neutron irradiation of tumor tissue combined with BPA in sorbitol solution

A U-87 MG brain tumor mouse model was created by subcutaneous injection of 2×10^6 U-87 MG cells into the left flank of 8-week-old Balb/cAJcl-nu/nu female mice (CLEA Japan, Tokyo, Japan). Those with palpable subcutaneous tumors 14 days after tumor injection were divided into three groups: a no treatment control group (n = 10), a neutron irradiation only group (n = 10), and a neutron + BPA group (n = 10). The mice were grouped to ensure homogeneous mean weights between test groups. A SAS squamous cell carcinoma mouse model was created by subcutaneous injection of 1×10^6 SAS cells into the left flanks of 8-week-old Balb/cAJcl-nu/nu female mice (CLEA Japan, Tokyo, Japan). Mice with palpable subcutaneous SAS tumors 6 days after tumor injection were grouped in the same manner as the U-87 MG mouse model. The SAS tumor model also used 10 mice per group.

One h after intravenous administration of 500 mg/kg of BPA in sorbitol solution into the tail vein, the mice were irradiated with neutrons using an accelerator-based neutron source (BNCT30, Sumitomo Heavy Industries, Ltd, Tokyo, Japan). The total BNCT dose was calculated according to IAEA-TECDOC-1223 [12]. Briefly, BNCT dose (Gy-eq) is calculated using the following equation:

(Eq.1.) BNCT dose [Gy-eq] = $C_B \times D_B \times CBE_B + D_N \times RBE_N + D_H \times RBE_H + D_\gamma \times RBE_\gamma$

where C_B is the boron concentration in the focal tissue, D_B is the boron-derived dose due to the $^{10}B(n,a)^7Li$ reaction per boron concentration in the tissue, CBE_B is the compound biological effective value, D_N is the nitrogen-derived dose due to the $^{14}N(n,p)^{14}C$ reaction, RBE_N is the relative biological effective value for the $^{14}N(n,p)^{14}C$ reaction, D_γ is the gamma-ray dose emitted from the neutron beam port and induced in the tissue by the $^1H(n, \gamma)^2H$ reaction, RBE_γ is the relative biological effective value for gamma-ray value. The RBE_N, RBE_H, and RBE_γ parameters used for dose calculation were 2.9, 2.4, and 1.0, respectively [13]. A CBE_B value of 2.5 was used for skin dose calculation [14]. The neutron irradiation time was set so the skin dose from BNCT was 4 Gy-eq. The neutron irradiation field was limited to the left lower limb of each mouse, where the tumors were implanted, and other areas were shielded using a lithium fluoride plate. Tumor volumes were measured 22 and 29 days after irradiation in the U-87 MG model and the SAS model, respectively.

2.8 Statistical analysis

Data were expressed as means ± standard deviations. Between groups differences in the *in vivo* experiments were analyzed using one-way analyses of variance with Dunnett's multiple comparison correction. A *p*-value (two-sided tests) < 0.05 was considered statistically significant. All statistical analyses were performed using Prism 9 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 BPA is more stable in sorbitol than fructose solution

Table 1 shows the long-term stability of BPA in fructose and sorbitol solvents at 60 °C. The solubility of BPA in water without a dissolving agent is 1.6 mg/mL. This rises to over 20 mg/mL when fructose or sorbitol is used as a dissolving agent. Thus, the solubility of BPA is dramatically increased by dissolving agents. After 4 weeks of storage at 60 °C, the BPA in fructose solution was a brownish color (indicative of a Maillard reaction) and decreases in the pH and osmotic pressure to saline ratio were found, in addition to a marked decrease in the BPA concentration. In contrast, the BPA in sorbitol solution showed only slight changes in its properties. There were no changes in pH or osmotic pressure to saline ratio, and only a slight decrease in BPA concentration (Table 1).

3.2 Pharmacokinetic results of intravenous and subcutaneous administration of ^{14}C-radiolabeled BPA to normal and tumor tissue in a tumor mouse model

Figure 2 shows the radioactive drug concentrations in the tumors and normal tissue of tumor-bearing

mice treated intravenously with ^{14}C-radiolabeled BPA at doses of 125 mg/kg or 500 mg/kg (Figs 2a–2d). The radioactivity of the tumor tissue in the 125 mg/kg group was 165, 150, and 101 μg eq./g at 0.25, 1, and 2 h, respectively. The mean tumor volumes and mean body weights of mice before drug administration did not differ significantly among different time points (0.25 h: 236.4 mm^3 and 25.2 g, 1 h: 247.7 mm^3 and 26.1 g, and 2 h 243.9 mm^3 and 24.9 g, respectively). In the 500 mg/kg group, the radioactivity was 388, 387, and 352 μg eq./g at 0.25, 1, and 2 h, respectively, and the mean tumor volumes and mean body weights of mice did not differ significantly (0.25 h: 236.4 mm^3 and 25.4 g, 1 h: 247.7 mm^3 and 25.7 g, and 2 h 243.9 mm^3 and 25.8 g, respectively). While blood drug concentrations decreased over time after administration in both dosage groups, this decrease was slower in tumor tissue than that in blood, suggesting that the drug was retained in the tumors.

Table 2 shows the drug concentrations in plasma and whole blood, and the plasma-to-whole blood ratio of drug concentrations, and the concentrations in blood cellular components calculated from Hct values. Plasma drug concentrations were 0.88 times lower than total blood drug concentrations regardless of the dose of BPA or the time since administration (Table 2). Drug concentrations in the cellular components of blood in the 500 mg/kg BPA dosage group were 424.1, 188.7, and 185.0 μg eq./mL at 0.25, 1 and 2 h, respectively. The drug concentrations in blood cellular components were higher than those in whole blood at all time points.

Figure 3 shows the radioactivity levels in the tumors and normal tissues of tumor-bearing mice after subcutaneous injection of ^{14}C-radiolabeled BPA at a dose of 500 mg/kg (Fig. 3a-b). The mean tumor volumes and mean body weights of mice did not differ significantly among different time points (1 h: 312.7 mm^3 and 24.4 g, 2 h: 288.1 mm^3 and 26.4 g, 3 h: 296.5 mm^3 and 24.6 g, and 4 h: 298.4 mm^3 and 27.0 g, respectively). The drug concentrations in the tumor tissue were 481, 419, 266, and 191 μg eq./g at 1, 2, 3, and 4 h post-administration, respectively. No obvious differences in drug concentrations were seen between tumor tissue from mice who received intravenous and subcutaneous drug administration. The plasma-to-whole blood drug concentration ratios in the subcutaneous mice dosed with 500 mg/kg were 0.92, 0.95, 0.99, and 1.04 1, 2, 3, and 4 h after administration, respectively. In addition to the organs measured in the intravenous administration experiments, the subcutaneous administration experiments also examined the pharmacokinetics of the drug in the pituitary gland, eyeball, small intestines, large intestines, and oral mucosa. The tissue of the small and large intestines, as well as that of the lung and liver, showed time-dependent decreases in drug concentrations, as did the blood. Interestingly, drug concentrations behaved differently in the oral mucosa than the other organs, being lower one h after administration and increasing 2 and 3 h after administration.

3.3 In vitro and in vivo antitumor effects of BNCT with BPA in sorbitol solution

Figure 4a and 4b show the tumor cell survival rates of U-87 MG and SAS cells after irradiation with neutrons in the presence of boron concentrations (10 mgB/ml and 25 mgB/ml, respectively). The

antitumor effects of BNCT were found to depend on both the concentration of boron in the medium and the neutron dose. Figure 4 also shows tumor size comparisons between tumor-bearing mice treated with 500 mg/kg BPA and irradiated with neutrons, those treated with neutron irradiation only, and those that received no treatment. Figures 4c and 4d present these comparisons for U-87 MG and SAS tumor-bearing mice, respectively. In both the U-87 MG and SAS mouse models, BPA treatment followed by neutron irradiation resulted in a significant decrease in tumor volume compared to both the untreated group and the neutron irradiation group ($p < 0.001$). In both mouse models (U-87 MG and SAS), there was no significant difference in mouse weight at the time of boron drug administration between treatment groups (U-87 MG: No treatment group = 22.43 ± 1.50 g, Neutron alone group = 22.34 ± 1.03 g, Drug + Neutron group 22.09 ± 1.03 g, SAS: No treatment = 21.6 ± 0.9 g, Neutron alone = 21.4 ± 1.0 g, Drug + Neutron = 21.1 ± 1.0 g).

4 Discussion

In this study, we found (1) that BPA is more stable in sorbitol solution than fructose solution over long periods; (2) that BPA in sorbitol solution accumulates in tumors and normal tissue in the same way as BPA in fructose solution; (3) that BPA in sorbitol solution with neutron irradiation has reliable antitumor effects both *in vitro* and *in vivo*. The demand for a stable BPA solution has arisen with the increasing use of BNCT in clinical practice. Since BPA in fructose solution has a shelf life of under 2 weeks, a more stable BPA solubilizer was required [15]. Against this background, a method was developed to

dissolve BPA using sorbitol, a sugar alcohol. This study showed BPA to be much more stable in sorbitol solution than in fructose solution when stored for long periods. In this pharmacokinetic study, we have shown the tissue accumulation of a sorbitol solution of BPA over time in the tumor and in each normal tissue (organs at risk in radiotherapy). In this study, instead of measuring boron atoms as a surrogate for BPA by means of inductively coupled plasma, the benzene ring was labeled with the ^{14}C-radioisotope to measure the structure of BPA itself, which has never been reported before. In addition, we demonstrated that a sorbitol solution of BPA also showed an antitumor effect after neutron irradiation.

We have previously reported the results of boron concentrations in normal tissues after subcutaneous administration of BPA in fructose solution to mice using an inductively coupled plasma method [16]. The inductively coupled plasma method measures boron atoms as an alternative to BPA itself based on the assumption that boron bound to the benzene ring of BPA (Fig. 1) is not displaced *in vivo*. If the boron atoms were to separate from the benzene ring *in vivo*, the pharmacokinetics of the drug would not be accurately evaluated using the inductively coupled plasma method. Because of these concerns, we conducted pharmacokinetic studies in this study using ^{14}C-radiolabeled BPA, in which the benzene ring of BPA was directly labeled with the ^{14}C radioisotope. The pharmacokinetic results of 500 mg/kg of subcutaneously administered BPA in a previous report [16] and in this study were as follows: the ratios of each tissue-to-blood concentration of the BPA fructose solution measured by the inductively coupled plasma 1 h after injection were 1.38 (skin), 0.43 (brain), and 0.82 (liver), whereas those of the BPA

sorbitol solution measured with ^{14}C-radiolabeled BPA in this study were 1.30 (skin), 0.41 (brain [cerebrum]), and 1.09 (liver). Upon comparing the results of this experiment to those of previous studies, no huge differences in the tissue-to-blood concentration ratios were observed. According to the disclosed data on the metabolites of BPA *in vivo* by the Pharmaceuticals and Medical Devices Agency in Japan [17], BPA is largely unaffected by metabolism *in vivo*, most of it is still present in an unchanged form of BPA 8 h after the administration, and few metabolites of BPA were observed in which the boron atom in BPA was separated from the benzene ring [17]. This result is consistent with the above comparison between the results of this study and those of previous studies in which BPA was measured using the inductively coupled plasma method.

The dose-limiting tissue of BNCT, especially in view of late effects, depends on the location of the tumor and how radiotherapy with X-rays has already been performed prior to BNCT, since in most of the cases, patients who had received BNCT experienced a relapse of cancer and they had already received radiotherapy as their first treatment. For malignant brain tumors, the standard initial treatment is surgery and chemotherapy combined with radiotherapy at the postoperative site. When these patients suffer from a relapse of cancer, some of them are introduced to BNCT treatment. In this case, since the brain has already received a large amount of radiation prior to BNCT, the dose-limiting tissues for BNCT for brain tumors are the brain and the skin. In the case of head and neck malignancies, as in the case with malignant brain tumors, BNCT is often performed for recurrent cases after radiotherapy or

chemoradiotherapy. Therefore, the dose-limiting tissues after BNCT in head and neck cancer are the skin, subcutaneous tissue, mucosa, blood vessels, and brain (when the irradiated lesion is close to the brain), especially in terms of late effects. In this experiment, we decided to irradiate mice with neutrons 1 h after drug administration for the following three reasons (based on the results of a pharmacokinetic study conducted using intravenous BPA): first, the boron concentration in the tumor remains high from 0.25 h to 1 h; second, the boron concentration in the blood is high in the early stage of administration, and then rapidly decreases over time; and third, the ratio of the boron concentration to tumor tissue in dose-limiting tissues does not differ significantly between 0.25 h and 1 h.

As with conventional X-ray radiotherapy, BNCT can be expected to have more antitumor effects if a higher dose is delivered to the tumor. However, as with X-ray radiotherapy, the dose that can be delivered in BNCT is defined by the dose to the surrounding normal tissues (organs at risk). One of the organs at risk in BNCT is the skin and surrounding mucosa in head and neck cancer and the brain and the skin in malignant brain tumors depending on the tumor lesion. In the present neutron irradiation experiment, irradiation conditions of 4 Gy-eq to the skin were used. This irradiation dose is less than that of conventional X-ray radiotherapy. The reason for this is that this irradiation experiment aimed to find via animal studies the minimum dose necessary to show a significant antitumor effect as a preliminary step to safely perform BNCT on patients. We also aimed to measure the tumor size and confirm the relatively long-term antitumor effect at the point of animal experiments for 1 month.

Therefore, it was necessary to conduct experiments under neutron irradiation conditions that would ensure the long-term survival of the experimental animals after irradiation. The results of this study showed that, at least in the mouse tumor model used in this study, significant inhibition of tumor growth was observed under the condition of 4 Gy-eq to skin irradiation, suggesting that BNCT with BPA in sorbitol solution has a tumor-suppressive effect after neutron irradiation at ≥4 Gy-eq to the skin.

In this study, we measured boron concentrations in whole blood, as well as boron concentrations and Hct values in plasma by separating the cellular components of the whole blood. This is because the minimum BNCT radiation dose received by each tumor cell depends on plasma boron concentrations [18,19]. The concentration of boron in the interstitial fluid within the tumor tissue is considered equivalent to the plasma boron concentrations. Even if some of the tumor cells have a weak boron uptake, sufficient boron in the interstitial fluid induces a neutron capture reaction in the interstitial fluid very close to the cells, and heavy particles are irradiated to those cells. Therefore, even with variations in the boron uptake capacity of tumor cells [20], the boron in the interstitial fluid can be expected to be delivered to the tumor cells. This is an important finding for the establishment of a BNCT dose calculation method that considers the distribution of boron agents in tumor tissues at the micro level.

We used 3.0 w/v% and 2.5 w/v% of BPA sorbitol solutions in this study. For stability experiments, we used 2.5 w/v% (0.12 mol/L) BPA sorbitol solution, which is close to the concentration of the fructose

solution (2.42 w/v%, 0.116 mol/L). This concentration of BPA in fructose solution is approximately the upper limit for BPA, considering the osmotic pressure to saline ratio in the physiologically acceptable range (2.5–3 in general) for infusion, where intravenous administration of drugs with high osmotic pressure causes phlebitis and pain during infusion. As shown in Table 1, the osmotic pressure to saline ratio was lower in sorbitol solutions of BPA than in fructose solutions under the same BPA concentration (mol/L) conditions. In addition to the poor stability of fructose solutions of BPA, their major clinical challenge is that the total volume of the infusion becomes relatively high when the required amount of BPA is administered intravenously. In this situation, there is a potential risk of occurrence of heart failure, especially for patients with cardiac diseases. Using sorbitol as a solvent allows for a higher concentration of BPA with physiologically acceptable osmotic pressure. Therefore, in clinical practice, a concentration of 3.0 w/v% is used for sorbitol solution of BPA. Thus, we used 3.0 w/v% of BPA sorbitol solution, the same concentration that is used clinically, for pharmacokinetic studies.

However, this study has one limitation. We used only female mice were used for the experiments. The impact of sex differences is important for assessing the validity of animal models used in preclinical studies. The main cause of the sex difference is the activity of enzymes involved in drug metabolism, such as cytochrome P450s, CYP2C11, CYP2C13, CYP3A2, and CYP2C12 [21]. Per previous reports, BPA is not affected by hepatic metabolizing enzymes and is excreted mainly via the urinary tract in its original form [17]. Therefore, we considered the effect of sex differences to be minimal.

5 Conclusion

In conclusion, we investigated the pharmacokinetics of BPA in sorbitol solution in mice using ^{14}C-radiolabeling and confirmed the antitumor effects of neutron irradiation after administration. The methods, antitumor effects, and detailed pharmacokinetics of BPA established in this study can provide a basis for the development of new BNCT boron drugs in the future.

Figure legends

Fig. 1 Schematic diagram of boron neutron capture therapy (BNCT).

a. ^{10}B atoms capture neutrons and induce their fission into two particles: alpha particles and ^{7}Li nuclei. The ranges of these alpha particles and lithium nuclei are less than <5 μm and <9 μm, respectively, — less than the typical diameter of a tumor cell (10–20 μm); b. Primarily cells that have incorporated boron atoms die as a result of nuclear reactions between neutrons and boron; c. Chemical structure of *p*-boronophenylalanine (BPA); d. Chemical structure of ^{14}C-radiolabeled BPA.

Fig. 2 Radioactive drug concentrations in the tumors and normal tissue of U-87 MG tumor-bearing mice treated intravenously with ^{14}C-radiolabeled BPA in sorbitol solution at doses of 125 mg/kg or 500 mg/kg.

a. Drug concentrations in each tissue type following BPA treatment at a dose of 125 mg/kg (n=9) ; b. Figure a scaled down to show kidney and pancreas values; c. Drug concentrations in each tissue type following BPA treatment at a dose of 500 mg/kg (n=9); d. Figure c scaled down to show kidney and pancreas values.

Fig. 3 Radioactive drug concentrations in the tumors and normal tissues of U-87 MG tumor-bearing mice treated subcutaneously with ^{14}C-radiolabeled BPA in sorbitol solution.

a. Drug concentrations found in each tissue type following subcutaneous administration of BPA in

445 sorbitol solution at a dose of 500 mg/kg (n=12); b. Figure a scaled down to show kidney and pancreas

446 values.

447

448 **Fig. 4 Antitumor effects of binary treatment with BPA in sorbitol solution and neutron irradiation**

449 **both *in vitro* and *in vivo*.**

450 a. The survival rates of U-87 MG tumor cells when irradiated with neutrons with boron concentrations

451 (10 ppm and 25 ppm) in the medium; b. The survival rates of SAS tumor cells when irradiated with

452 neutrons with boron concentrations (10 μgB/ml and 25 μgB/ml) in the medium; c. A comparison of

453 tumor volumes in U-87 MG tumor mice 29 days after BPA injection and neutron irradiation (n=10),

454 neutron treatment only (n=10), or no treatment (n=10). U-87 MG neutron vs BNCT: Hedges' g effect

455 size 2.23 (95% CI 1.15–3.47); d. A comparison of tumor volumes in SAS tumor mice 22 days after BPA

456 injection and neutron irradiation (n=10), neutron treatment only (n=10), or no treatment (n=10). SAS

457 neutron vs. BNCT: Hedges' g effect size 3.16 (95% CI 1.90–4.67) P-values of <0.05 were considered to

458 be statistically significant (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

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Statements and declarations

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Conflict of interest statement: Three of the authors (TY, TH, and KU) declare conflicts of interest based on their relationships with Stella Pharma Corporation. The remaining authors have no conflicts of interest to declare.

Author contributions: KO, TH, and KU conceived and designed the study. TY, HT, YK, GK, Shin-ichiro Masunaga, Suzuki Minoru performed experiments and data analyses. TW performed additional data analyses. KO, and Suzuki Minoru supervised the project. TW, TH, and KU wrote the manuscript. All authors edited and approved the manuscript.

Ethics approval: The mice used in this study were cared for and treated according to the Recommendations for the Handling of Laboratory Animals for Biomedical Research by the Committee on Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. Additionally, all animal experiments were approved by Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University (Approval number: 200813) and conducted per the laboratory animal handling guidelines of our institution.

Consent to participate: Not applicable

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Availability of data and materials: The raw data before analysis can be downloaded as supplementary data in this manuscript.

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