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ortho-Carboranyl Glycosides for the Treatment of Cancer by Boron Neutron Capture Therapy

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Abstract—Distinct biological properties of the *ortho*-carboranyl (1,2-dicarba-*closo*-dodecaboranyl) glycosides **1**, **2** and **3** were evaluated to estimate the suitability of these compounds for cancer treatment by boron neutron capture therapy. The boron uptake into B16-Melanoma cells was significantly higher by incubating the cells with aqueous solutions of carboranyl glucoside **1** (11.2 ppm after 3 h), lactoside **2** (13.2 ppm after 12 h) and maltoside **3** (20.0 ppm after 24 h) compared with solutions of clinically used *p*-boronophenylalanine (BPA) **5** (3.1 ppm after 24 h). Carboranyl maltoside **3** was more effective than boron-10 enriched **5** in killing C-6 rat glioma cells by incubating the cells with the compound and subsequent treatment with thermal neutrons. **3** was also administrated iv, in concentrations of 25 mg boron/kg body weight to rats bearing brain tumors. After a period of 4 h after administration the concentration of boron in the tumor tissue was 3.0 ppm.

Introduction

The boron neutron capture therapy (BNCT) for the treatment of cancer gained intense interest in recent years.[1–4] This therapy is based on the distinct property of ^{10}B to react with slow neutrons to give $^{7}Li^{3+}$ and $^{4}He^{2+}$ in a nuclear reaction; if boron is present in a cell, this reaction causes a strong cytotoxic effect. However, the success of neutron capture therapy depends on the selective deposition of a sufficient amount of boron in the tumor tissue (20–30 μg B/g tissue[5]). Most compounds hitherto developed for this kind of therapy are not suitable due to their low water solubility, stability and low selectivity towards cancer cells. Moreover, many of the reported compounds display a high toxicity. Recently we prepared the carboranyl glucoside **1** and the even better water-soluble lactoside **2** and maltoside **3**[6] (Scheme 1). These compounds contain the *ortho*-carborane (1,2-dicarba-*closo*-dodecaborane) unit **4** which is stable in aqueous media. In addition, one molecule is capable of transporting ten boron atoms into the cells. Moreover, we also have synthesized fluorinated analogues of **1** and **2** to allow an easy detection of these compounds in tumor tissue by NMR spectroscopy.[7] We have already reported[6,7] that the carboranyl glycosides display almost no cytotoxicity up to concentrations of 0.40 mM on human bronchial carcinoma cells of the line A549, if the cells are incubated with the compounds in a medium without glycohydrolase activity.

In this paper we report in vitro as well as in vivo experiments to evaluate the applicability of the new carboranyl glycosides **1**, **2** and **3** for cancer treatment by boron neutron capture therapy. For comparison, *p*-boronophenylalanine (BPA) **5**, which is clinically used for neutron capture therapy of human melanoma[8] as well as mercaptoundecahydro-dodecaborate (BSH) **6**[9] were also included in these investigations.

The carboranyl glycosides **1**, **2** and **3** were readily prepared starting with glycosidation of propargyl alcohol or 3-butyn-1-ol with the trichloroacetimidates[10] of glucose **7**, maltose **8** and lactose **9** to give the alkynes **10**, **11**, and **12**, respectively. Subsequent addition of decaborane(14) to the triple bond with formation of **13**, **14**, and **15**, respectively, followed by deprotection led to the desired products (Scheme 2).[6]

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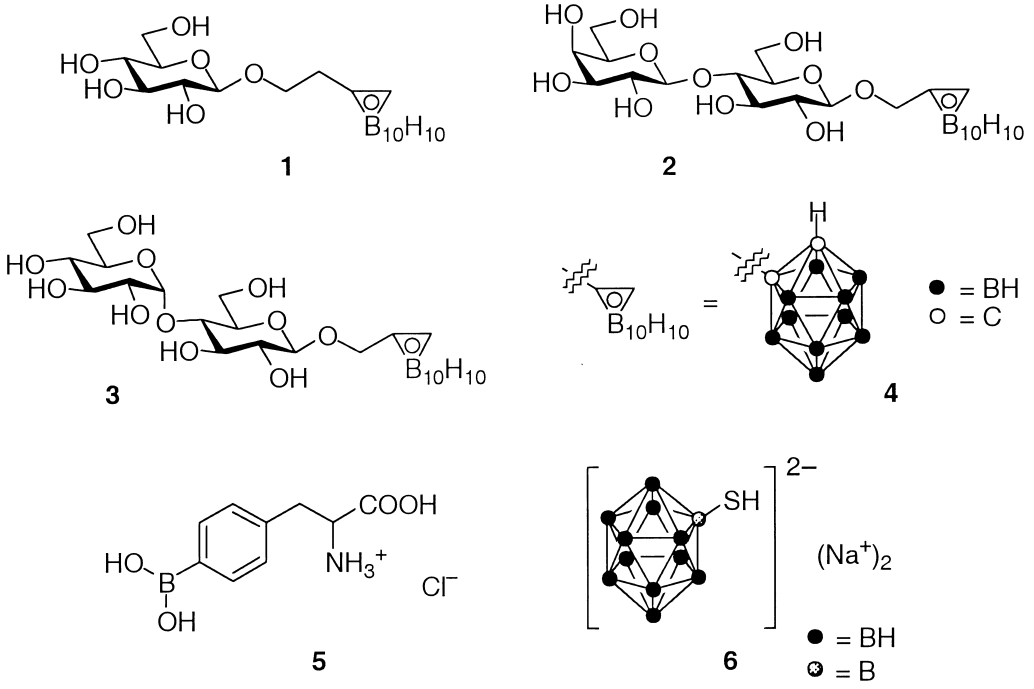
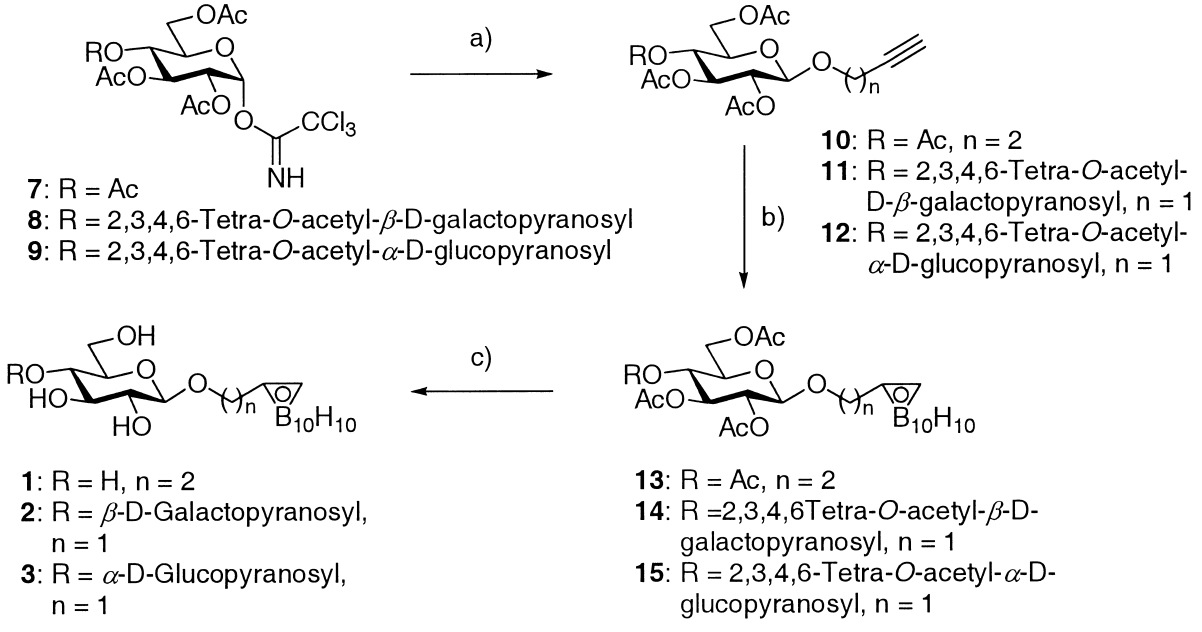
Results

In vitro boron incorporation into B-16 melanoma cells

The results of the in vitro boron incorporation into B-16 melanoma cells are presented in Figure 1. Carboranyl maltoside **3** exhibited highest uptake by B-16 cells among the carboranyl glycosides described in this paper. The boron concentration in B-16 cells at 3 h after administration was 6.1 ppm and increased up to 20.0 ppm after 24 h. In the case of lactoside **2**, the boron concentration was 11.7 ppm at 3 h and reached a maximum value (13.2 ppm) at 12 h. The boron concentration of glucoside **1** in the cells reached a maximum value (11.2 ppm) at 3 h and then gradually decreased. How-



Scheme 1. Structures of the carboranyl glucoside **1**, lactoside **2** and maltoside **3** with the *o*-carboranyl subunit **4**; structures of BPA·HCl **5** and of BSH **6**.



Scheme 2. Synthesis of carboranyl glycosides **1**, **2** and **3**. Reagents: (a) **10**: 3-butyn-1-ol, CH_2CH_2, $BF_3{\cdot}OEt_2$, 0 °C to rt, 55%; **11**: propargyl alcohol, CH_2CH_2, $BF_3{\cdot}OEt_2$, 0 °C to rt, 66%; **12**: propargyl alcohol, CH_2CH_2, TMSOTf, 0 °C, 53%; (b) **13**: $B_{10}H_{14}$ in CH_3CN, reflux, then **10** in toluene, reflux, 61%; **14**: $B_{10}H_{14}$ in CH_3CN, reflux, then **11** in toluene, reflux, 43%; **15**: $B_{10}H_{14}$ in CH_3CN, reflux, then **12** in toluene, reflux, 49%; (c) **1**: NaOMe, MeOH, rt, 97%; **2**: NaOMe, MeOH, 71%; **3**: NaOMe, MeOH, rt, 71%.

ever, low concentrations (1.4–3.1 ppm) in the cells were observed with BPA·HCl **5**.[8]

In vitro boron incorporation into C6 rat glioma cells

Since the carboranyl maltoside **3** exhibited the highest uptake into B-16 melanoma cells, further experiments were performed with this compound.

Figure 2 indicates that the boron accumulation in C6 rat glioma cells achieved by incubating the cells in culture media containing various concentrations of **3** (5, 10 and 15 ppm) was dose-dependent. Figure 3 shows the time-dependent uptake by C6 cells. The boron concentration reached a maximum value (65.7 ppm) at 12 h after administration of the compound and then gradually decreased.

In vitro survival study of C6 glioma cells after thermal neutron irradiation

Figure 4 shows the survival fraction of the cells after the thermal neutron irradiation. The thermal neutron beam had a significant killing effect on C6 glioma cells which were exposed to solution of carboranyl maltoside **3** before. Although **3** contained natural boron, 15 ppm of boron loading in the cells by **3** was more effective than 10 ppm of BSH **6**,[9] which was used in boron-10 enriched form.

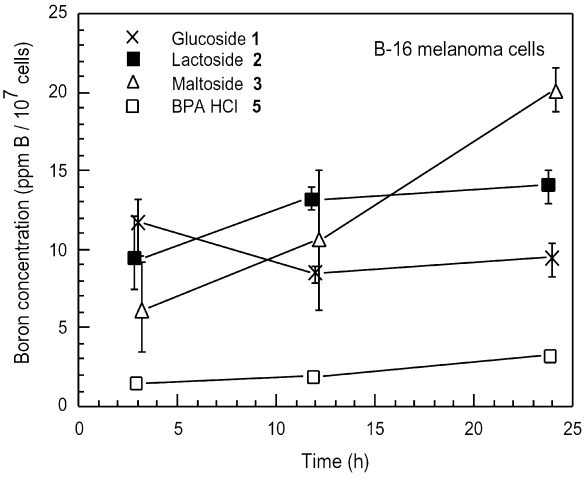


Figure 1. Boron incorporation into B-16 melanoma cells. The cells were incubated for 3, 12 and 24 h with Eagle-MEM medium containing the carboranyl glucoside **1**, lactoside **2** and maltoside **3**, respectively (boron concentration: 10.8 ppm, 1.0×10^{-4} M). Each point represents the mean ± SE of triplicate experiments.

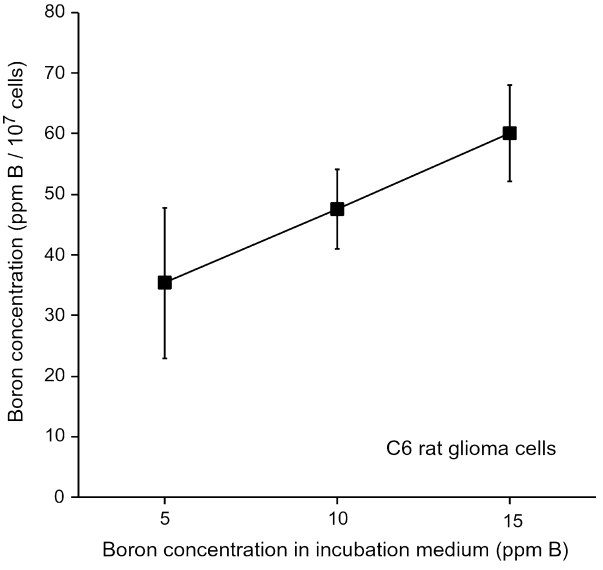


Figure 2. Boron incorporation into C6 rat glioma cells. The cells were incubated for 6 h with Eagle-MEM medium containing various concentrations of carboranyl maltoside **3** (boron concentration: 5, 10 and 15 ppm). Each point represents the mean ± SE of triplicate experiments.

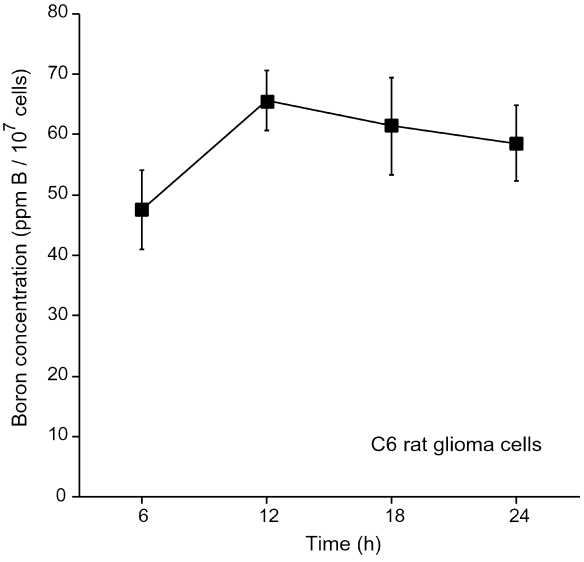


Figure 3. Boron incorporation into C6 rat glioma cells. The cells were incubated for 6–24 h with Eagle-MEM medium containing carboranyl maltoside **3** (boron concentration: 10 ppm). Each point represents the mean ± SE of triplicate experiments.

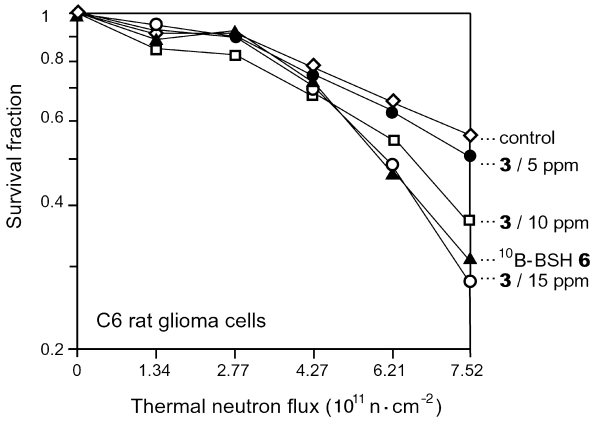


Figure 4. In vitro survival study of C6 rat glioma cells after thermal irradiation. The cells were incubated for 6 h with Eagle-MEM medium containing various concentrations of carboranyl maltoside **3** (boron concentration: 5, 10 and 15 ppm) and irradiated with thermal neutrons at a neutron flux of 1.95×10^{9} n cm^{-2} s^{-1}.

In vivo boron uptake study

Figure 5 shows the time-dependent boron concentration in blood, brain tumor and normal brain tissues after the intravenous administration of carboranyl maltoside **3** and Figures 6 and 7 display those in the surrounding organs of the brain (scalp, eye, temporal muscle) and those in the viscera (kidney, liver, intestine, urinary and lung).

The maximum boron levels in tumor and blood were observed at 1 h after the administration of **3**, whereas in the normal brain tissue a maximum boron concentration was observed after 4 h (Fig. 5). The changes in the boron concentration in the surrounding tissues of the brain showed the similar tendency; the maximum levels were observed at 1 h after the administration and gradually decreased (Fig. 6). In the abdominal viscera, liver and kidney the boron concentration was much higher than that of the blood (Fig. 5 versus Fig. 7). It should be noted that the in vivo study showed some side effects of **3** in the rats. Most of the rats manifested haematuria within 1 h after the intravenous administration of this compound, but none of the rats died during the experiment.

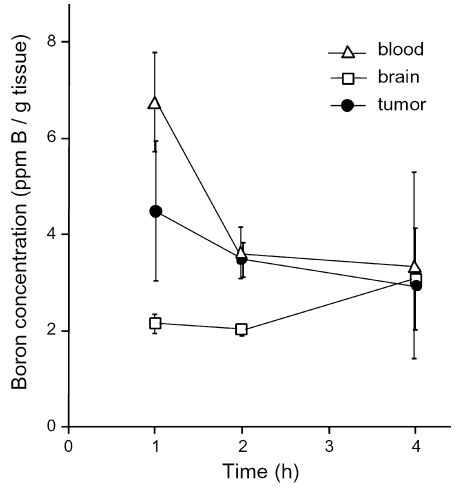


Figure 5. The distribution of boron incorporated into blood, brain and tumor tissues of the tumor bearing rats after injection of carboranyl maltoside **3** (25 mg of boron per kilogram of the body weight). Each point represents the mean ± SE of triplicate experiments.

Discussion

The boron compound used for clinical application of BNCT must give a suitable biodistribution in vivo and must also be safe. In our in vitro study, carboranyl maltoside **3** was incorporated into B-16 melanoma as well as into C6 glioma cells resulting in favorable boron concentrations in the cells. The maximum boron concentrations were 20.0 ppm in B-16 cells at 24 h and 47.7 ppm in C6 cells at 6 h after administration of the compound. In comparison, administration of the clinically used BPA·HCl **5** under identical conditions led to a boron concentration of 1.4–3.1 ppm in B-16 melanoma cells. Thermal neutron irradiation study also suggested a good potential of carboranyl maltoside **3** as a boron carrier. A thermal neutron beam had a high killing effect on C6 glioma cells, which were incubated with **3** before. Although the compound contained natural boron, 15 ppm of boron loading by **3** was more effective than 10 ppm of BSH, which was used in boron-10 enriched form. As expected, the in vivo biodistribution study showed a lower uptake of **3** by the brain tumor tissue. The boron concentration achieved in the brain tumor 4 h after the iv administration of **3** was 3.0 ppm. In the in vivo study **3** caused some side effects in the rats. None of the rats died, however most of them

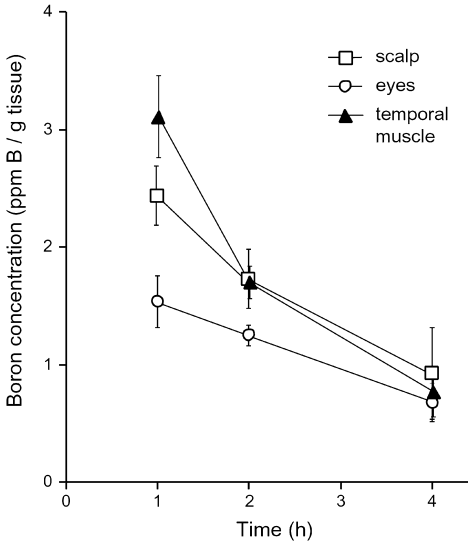


Figure 6. The distribution of boron incorporated into scalp, eyes and temporal muscle tissues of the tumor bearing rats after injection of carboranyl maltoside **3** (25 mg of boron per kilogram of the body weight). Each point represents the mean ± SE of triplicate experiments.

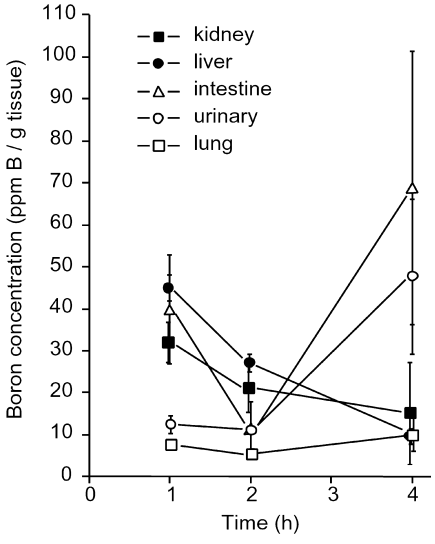


Figure 7. The distribution of boron incorporated into kidney, liver, intestines, urinary and lung tissues of the tumor bearing rats after injection of carboranyl maltoside **3** (25 mg of boron per kilogram of the body weight). Each point represents the mean ± SE of triplicate experiments.

manifested haematuria within 1 h after the iv administration of **3**. Since the histopathological examination of the urinary system of the rats was not fully performed, we cannot reach to a definitive conclusion, however, the examination suggested a vascular deterioration in the rats. This may be due to a 'tenside effect' of **3**. Were are therefore in the process of developing new compounds with a partial blocking of the hydroxyl goups of the sugar moiety.

Experimental

Tumor cells for in vitro evaluation

B-16 melanoma and C6 rat glioma cell lines were used in the biological study. B-16 cells were maintained in Eagle's MEM (Nissui Pure Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (JRH biosciences) and 1% Antibiotic-Antimycotic 100X (Gibco BRL). C6 cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (ICN Biochemicals Japan Co., Ltd., Tokyo, Japan), gentamicin sulfate (50 mg/L) and amphotericin B (250 mg/L).

In vitro boron incorporation into B-16 melanoma cells

B-16 Melanoma cells were cultured in Falcon 3025 dishes (150 mm ∅). When the cells were grown to fill up the dish, the cell number was counted ($4.0–5.0\times10^6$ cells/dish). Since all glassware and the cells themselves contain small traces of boron, one dish was used for control experiment without any of the synthesized compounds being added. The carboranyl glycosides **1**, **2** and **3** (1.0×10^{-4} M, 10.8 ppm B) and BPA (1.0×10^{-3} M, 10.8 ppm B) were added to the dishes with the cells being incubated for 3, 12 and 24 h at 37 °C in 20 mL of the medium (Eagle-MEM, 10% FBS). The cells were washed three times with Ca-Mg free phosphate buffered NaCl Solution (PBS(−): NaCl [137.0 mM], KCl [2.7 mM], Na_2HPO_4 [8.1 mM], KH_2PO_4 [1.12 mM]), collected, digested with a mixture of 0.7 mL of 60% $HClO_4$ and 1.3 mL of 30% H_2O_2 and then decomposed for 1 h at 75 °C. After filtration with membrane filter (Millipore, 0.22 μm), the boron concentration was determined by using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Shimadzu, ICPS-1000-III). The boron concentration of the control experiment was subtracted from the boron concentrations of the cells of each dish. Each experiment was carried out in three replications.

In vitro boron incorporation into C6 rat glioma cells

The cells in growth phase prepared in cell culture dishes were washed three times with PBS(−) and reincubated in the culture media containing various concentrations of carboranyl maltoside **3** (5, 10 and 15 ppm). After the incubation for 6 h, the cells were washed four times in PBS(−) and the number of the viable cells was counted using the dye-exclusion method. For the studies on time-dependency of the boron uptake, various incubation times (6, 12, 18 and 24 h) were tested at a boron loading of 10 ppm. The cells were centrifuged again and supernatant was removed. Then the cells were chemically digested with a mixture of 0.15 mL of 70% $HClO_4$ and 0.3 mL of 30% H_2O_2 at 75 °C. After 1 h at 75 °C, the samples became colorless and were diluted with 2.0 mL of distilled water and filtered through a 0.45 μm filter. Boron concentration of the samples was measured using ICP-AES (VR1200, SEIKO Electric Co., Fukuoka, Japan) and the cellular boron uptake was calculated based on the measured boron concentration of the digested samples and the number of tumor cells contained in the samples (estimated to 1.0×10^9/g).

In vitro thermal neutron irradiation

C6 in growth phase was prepared in the cell culture dishes and reincubated in the culture media containing various concentrations of carboranyl maltoside **3** (5, 10 and 15 ppm). After the incubation for 6 h, the cells were trypsinized, washed four times in PBS(−), and a cell suspension of 2.0×10^4 cells/mL was prepared. One mL of the suspension in column-shape teflon tubes was irradiated with thermal neutrons at Kyoto University Research Reactor (KUR) at a neutron flux of 1.95×10^9 n cm^{-2} s^{-1}. After the thermal neutron exposure, a colony formation assay was performed in a routine manner to evaluate the survival fraction of the cells.

Brain tumor model for in vivo evaluation

For in vivo biodistribution study, tumor-bearing rats were experimentally produced using an intracranial transplantation technique of C6 cells. Nine three-week-old male Wistar rats (Kyudo, Fukuoka, Japan) were fed ad libitum and allowed free access to water throughout the experiments. Under pentobarbital anesthesia (ip, 40 mg/kg), the rats were fixed to a stereotaxic frame and received a midline incision of the head. After the exposure of the skull, a burr hole on 2 mm to the right of the midline along the coronal suture was made by a dental drill. The suspension of C6 cells [5.0×10^6 cells/head, 10 μL in PBS(−)] was injected into nucleus caudate in the right hemisphere of the brain through a 23-gauge needle and a microsyringe. After the needle was withdrawn, the burr hole was closed with bone wax and the skin was closed with surgical clips.

In vivo uptake study

Twelve days after the tumor transplantation, the tumor-bearing rats were subjected to iv administration of the boron compound. Carboranyl maltoside **3** was dissolved in sterile NaCl solution and 25 mg/kg of boron was slowly given intravenously to each rat via tail vein. Each three rats were sacrificed at 1, 2 and 4 h after the administration with an overdose of pentobarbital (ip) and weighted samples ranging 200 to 400 mg were obtained from the following tissues: blood, brain tumor, normal brain, scalp, eye, temporal muscle, kidney, liver, intestine, urinary and lung. The boron concentrations achieved in these 11 samples were measured by the ICP-AES method as described above.

Acknowledgements

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