

Hydroxylforms of *p*-Boronophenylalanine as Potential Boron Carriers on Boron Neutron Capture Therapy for Malignant Brain Tumors¹

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Abstract

Hydroxylforms of boronophenylalanine (BPA) were synthesized by conjugation with a cascade of polyols to decrease the BPA uptake of normal parenchyma without affecting uptake into the tumor. We determined their tumor cell killing effect on boron neutron capture therapy (BNCT) against BPA using the human glioma cell line T98G. The thermal neutron doses yielding the D_{37} (dose used to inhibit 63% colony formation) values of *dl-p*-BPA(OH)_{*n*} were 1.45×10^{12} nvt ($n = 1$), 1.33×10^{12} nvt ($n = 2$), 3.37×10^{12} nvt ($n = 4$), and 1.72×10^{12} nvt ($n = 0$). The relative tumor cell killing effect on BNCT of *dl-p*-BPA(OH)_{*n*} against *dl-p*-BPA, which was defined as the ratio of D_{37-BPA} to $D_{37-BPA(OH)_n}$, was 1.18 ($n = 1$), 1.29 ($n = 2$), and 0.51 ($n = 4$). The tumor:normal brain ratio of *dl-p*-BPA(OH)_{*n*} in 9L rat brain tumor models was improved 1.2- ($n = 1$) and 1.4-fold ($n = 2$) against that of *dl-p*-BPA without a decrease of its uptake into the tumor. The water solubility of BPA(OH)_{*n*} increased against BPA, and the toxicity represented as the IC_{50} value of *dl-p*-BPA(OH)_{*2*} was nearly one half that of *dl-p*-BPA, being established in our previous works. Hydroxylforms of BPA, especially *dl-p*-BPA(OH)_{*2*}, might be more suitable boron carriers of BNCT to malignant brain tumors since the radiation injury to the normal parenchyma surrounding the tumor is reduced.

Introduction

Clinical trials of the BPA³-based BNCT of malignant brain tumors have been investigated in the United States and Japan (1, 2). BPA should accumulate in brain tumor cells as a nonspecific amino acid derivative. The most convenient feature of BPA as a boron carrier for BNCT on malignant brain tumors is its T:B ratio of around 3 (3, 4), which is about 2–3-fold that of sodium borocaptate (5–8).⁴ The T:N ratio of this compound in the brain, however, is still too low for BNCT use, since it may cause radiation-induced necrosis of the related normal parenchyma surrounding the tumor. The T:N ratio of *l-p*-BPA is reported as 3–4 (3). This value does not allow deeply seated brain tumors to be treated because of safety reasons. Another problem of BPA-based BNCT is its low aqueous solubility, since BPA is applied as its hydrochloride, which has low solubility in water. To increase the T:N ratio and the water solubility of *l-p*-BPA itself without significantly affecting uptake into tumors, hydroxylforms of BPA have been

synthesized by Nemoto *et al.* (10) based on these basic concepts: (a) the permeability of the blood-brain barrier is dependent on the water solubility of a reagent and (b) the permeability of BPA through blood-brain barrier should be decreased by modifying its chemical structure, since a number of specialized mediated transport systems allow the transmission of certain amino acids (especially precursors to neurotransmitters). In this study, we investigated the effect of the hydroxylforms of BPA derivatives on BNCT using the human glioma cell line T98G. These compounds warrant further clinical study.

Materials and Methods

Synthesis of Hydroxylforms of BPA. Polyols of the cascade type (Fig. 1, 1), as a water-solubilizing element, have been developed for BNCT. Cascade polyols have no asymmetric center; therefore, no diastereoisomers are formed when they are bonded to biologically active molecules containing boron. Furthermore, the number of hydroxyl groups can be manipulated. 1,3-bis(benzyloxy)-2-propanol (Fig. 1, 5a) was converted to the corresponding tosylate 5b upon exposure to tosyl chloride/4-(dimethylamino)pyridine in pyridine. Treatment of 5b with sodium azide in DMF gave the azide derivative 5c. Reduction of 5c with LiAlH₄ in ether afforded 2-amino-1,3-bis(benzyloxy)-propane (Fig. 1, 5d). The overall yield of 5d from 5a was 95%. Similarly, 1,3-bis(1,3-dibenzyl-2-glyceroxy)-2-propanol (Fig. 1, 8a) was converted to the amine derivative 6d in 73% overall yield. BPA was converted to the Cbz-protected form 7, upon treatment with CbzCl in NaOH, in 98% yield. Treatment of 7 with 2 equivalents of *N*-methyl-diethanolamine in DMF gave the boronate 8, in which the B(OH)₂ group was protected by the *N*-methyl-ethanolamine moiety. Reaction of 8, without isolation and purification with ethanolamine in the presence of HOBt and EDC afforded 9, which was transformed to the monohydroxy borophenylalanine derivative 2, BPA(OH), upon hydrogenation with a Pd(OH)₂-C catalyst. The overall yield of 2 from 7 was 28%. Treatment of 8 with 5d under the same conditions as above (HOBt-EDC) produced 10 in 95% yield. The direct reaction of 7 with 5d in the presence of HOBt-EDC gave 10 in a lower yield (48% yield). Removal of the Cbz and Bn groups from 10 using H₂/Pd(OH)₂-C afforded 3, BPA(OH)₂, in 56% yield. A similar procedure was used to convert 8 with 6d. Without purification, 11 was converted to 4 upon hydrogenation. The tetrahydroxy BPA derivative 4, BPA(OH)₄, was obtained in 37% overall yield from 7. The chemical details are described by Nemoto *et al.* (10, 11).

In vitro Survival Study. A cell suspension of the human glioma cell line T98G in the logarithmic growth phase was prepared in Eagle's MEM (Nissui Co., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and 2 mM *l*-glutamine. Kanamycin was added at a concentration of 100 mg/liter. The cells were incubated in five culture dishes (100-mm tissue culture dishes; Corning Glass Works, Corning, NY) at a subconfluent concentration of $5 \times 10^4/10$ ml/dish, respectively, overnight at 36°C in a 5% carbon dioxide atmosphere. Various amounts of *dl-p*-¹⁰BPA(OH)_{*n*} ($n = 1, 2, \text{ and } 4$) and *dl-p*-¹⁰BPA · HCl (Boron Biologicals Inc., Raleigh, NC) were dissolved in MEM (FCS–), and a ¹⁰B concentration of 440 ppm was obtained by prompt γ -ray PGS (12) after balancing with MEM (FCS–). After exchanging the medium in the dishes with 10 ml MEM(FCS+), 455 μ l BPA solution disinfected by membrane filtration or the same amount of boron-free MEM (FCS–) were added. The pH of the medium mixed with *dl-p*-BPA · HCl was kept at 7.4 after balancing with a supplement with

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³ The abbreviations used are: BPA, boronophenylalanine; BNCT, boron neutron capture therapy; T:B, ¹⁰B concentration ratio in tumor:brain; T:N, ¹⁰B concentration ratio in tumor:normal brain; HOBt, *N*-hydroxybenzotriazole; EDC, ethyl-*N,N*-(dimethyl-3-aminopropyl)-carbodiimide; PGS, prompt γ -ray spectrometry; ATA, α track autoradiography; N:B, ¹⁰B concentration ratio in normal brain:brain.

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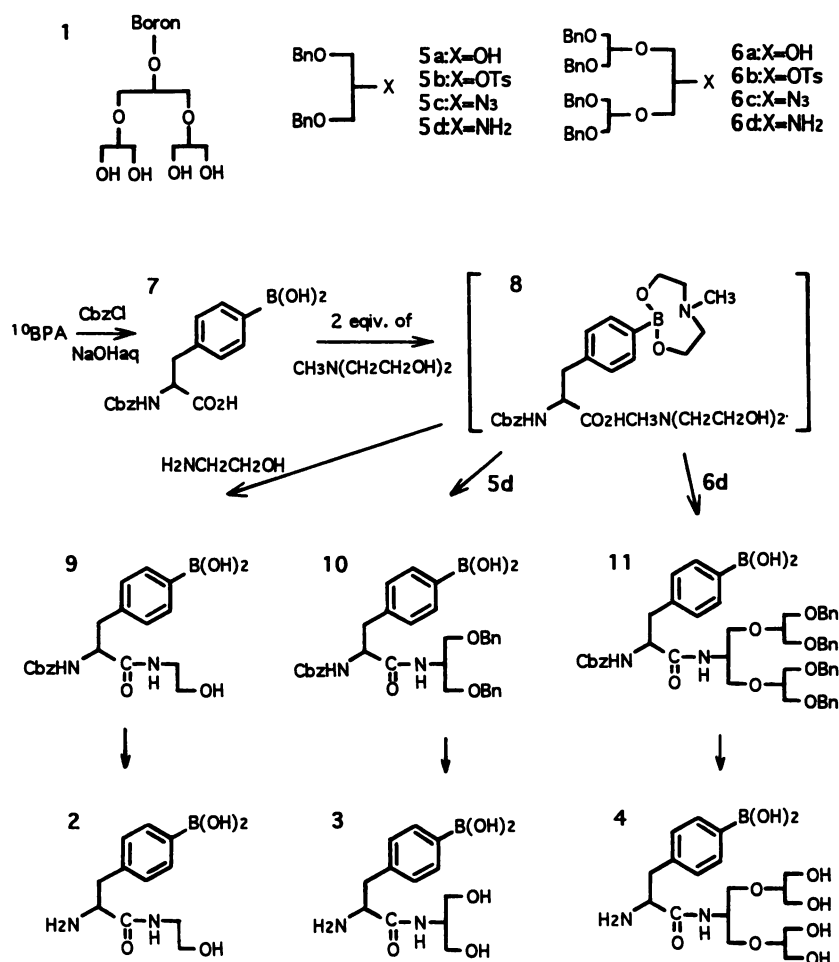


Fig. 1. Synthesis of BPA derivatives, *dl-p*-BPA(OH)_n (*n* = 1, 2, and 4), bearing cascade polyols. Details are described by Nemoto *et al.* (11).

NaHCO₃. ¹⁰B (20 ppm) in the medium was reconfirmed by PGS. Seven h after 20 ppm ¹⁰B loading, the cells were trypsinized and washed three times in boron-free MEM (FCS-), and 5 × 10³ cells/ml MEM (FCS+) were irradiated with thermal neutrons in column-shape Teflon tubes (1 × 3 cm). The cells did not adhere to the tubes, and no secondary radiation was caused by bombardment with the thermal neutrons. The thermal neutrons flux was 7.4 × 10⁹ cm⁻²s⁻¹, and the fluence range was 0, 0.44, 1.1, 2.2, 3.3, 4.4, 6.7, and 8.9 × 10¹² cm⁻². The thermal neutron fluence was determined by averaging two gold foils symmetrically attached to the surface of the Teflon tube along the direction of incidence of thermal neutrons. The γ-ray dose rate, including secondary γ-rays, was 10.1 cSv/min, according to a thermoluminescence dosimeter attached to the surface of a Teflon tube containing 1 ml MEM. After thermal neutron exposure, 300 or 900 cells were placed in three Corning 60-mm tissue culture dishes containing 6 ml MEM to examine colony formation. Ten days later, the colonies were fixed with formaldehyde and stained with 0.1% crystal violet for quantitative visualization by the naked eye. Values are represented as means ± SE. Three replications of this *in vitro* BNCT experiments were performed. *In vitro* BNCT was also performed on Greene's melanotic melanoma cells (13) that spontaneously arose in a Syrian golden hamster using *dl-p*-BPA and *dl-p*-BPA(OH)₂ under the conditions described above.

Assessment of the T:N Ratio in the Rat Brain Tumor Model. Rats were intracranially implanted with 9L gliosarcoma cells. Fisher 344 rats (7-week-old males, approximately 170 g body weight) were anesthetized by means of an i.p. injection of 0.1 ml/kg sodium pentobarbiturate (Nembutal). A tiny burr hole was made on the right parietal region 2 mm lateral and/or caudal to the Bregma with a high-speed drill. The 9L cells (10⁵/10 μl MEM (FCS+)) were slowly injected into the subcortical region to a depth of 3 mm from the dural surface using a 27-gauge Hamilton syringe. The burr hole was closed under sterile conditions using bone wax immediately after the removal of the syringe. Two weeks later, four types of BPA, *dl-p*-¹⁰BPA(OH)_n (*n* = 1 and 2),

dl-p-BPA · HCl, and *l-p*-BPA · HCl were dissolved in water at a concentration of 200 mg/ml, and 0.5 ml was given by p.o. administration, using a 23-gauge catheter, to five 9L rats under Nembutal sedation (0.05 mg/kg i.p.). Seven h later, the rats were guillotined and the whole brain, liver, and blood were removed and immediately frozen in liquid nitrogen for PGS. Frozen sections of the brain and the liver were prepared with a thickness of 10 μm. The sliced tissues were mounted on an α track detector (Kodak CN-85) and exposed to thermal neutrons at a flux of 3.2 × 10⁸ cm⁻²s⁻¹ for 45 min. The detectors were etched in 2.5 N NaOH at 60°C for 15 min, and α tracks originating in ¹⁰B in tissue grew into the etched pits that could be quantified under light microscopic observation in a field of 0.125 × 0.125 mm² under a magnification of 10 × 40 (14). The T:N ratio was calculated from the etched pit density ratio of the tumor in the right hemisphere against the left hemisphere (normal brain) on five 9L rats. The density of the etched pits is represented as the means ± SE on 10 microscopic fields after subtracting the background counts obtained from normal brain of a rat that did not receive boron. The ¹⁰B concentration in the normal brain (a left hemisphere) of 9L rats was determined by PGS. We calculated the T:B ratio, therefore, by multiplying the T:N ratio determined by ATA and the N:B ratio determined by PGS, since the brain tumors were too small to obtain reliable ¹⁰B concentrations by our PGS.

Results and Discussion

Fig. 2 shows the surviving fraction of T98G cells after the *in vitro* BNCT. No significant difference in the numbers of the cells among the loaded boron compounds was evident immediately after boron loading, and there were no apparent morphological changes. The plating efficiency for the colony formation was 68 ± 5%. The surviving fraction exponentially decreased without a shoulder. The thermal neutron doses yielding the D₃₇ (dose used to inhibit 63%

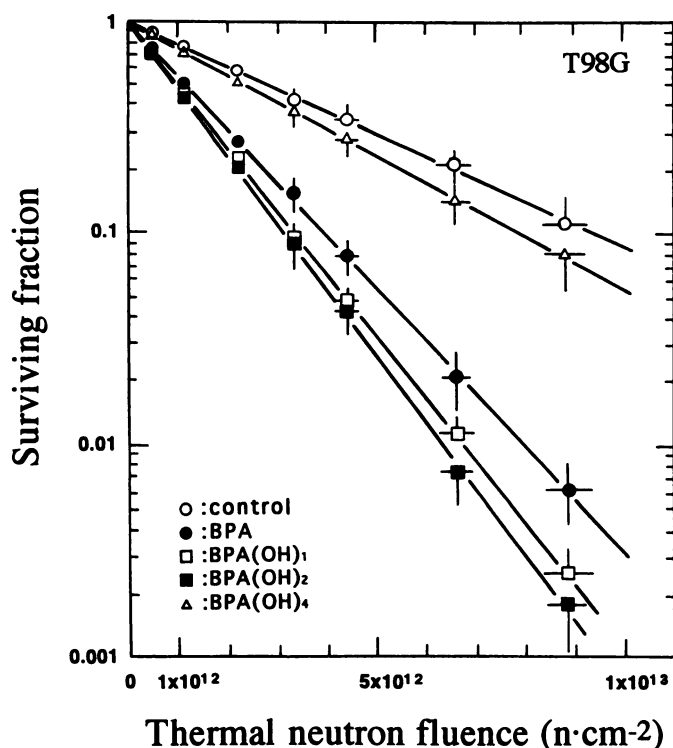


Fig. 2. The surviving fraction of T98G cells after *in vitro* BNCT. Values are means \pm SE.

colony formation) values of BPA(OH)_n were 1.45×10^{12} nvt ($n = 1$), 1.33×10^{12} nvt ($n = 2$), 3.37×10^{12} nvt ($n = 4$), and 1.72×10^{12} nvt ($n = 0$). The relative tumor cell killing effect on BNCT of *dl-p*-BPA(OH)_n against *dl-p*-BPA, which was defined as the ratio of D_{37-BPA} to $D_{37-BPA(OH)_n}$, was 1.18 ($n = 1$), 1.29 ($n = 2$), and 0.51 ($n = 4$). The D_{37} values for BPA(OH)_n ($n = 1$ and 2) were both lower than that of BPA ($P < 0.01$) and that of BPA(OH)₂ was slightly lower than that of BPA(OH)₁ ($P < 0.05$). The D_{37} value of BPA(OH)₄ was nearly twice that of BPA, and it showed no significant difference with that of the control ($P < 0.05$). The lower uptake of BPA(OH)₄ into B-16 melanoma cells and/or TIG-1-20 normal fibroblasts was also found in our previous studies (11). Although the water solubility of BPA(OH)₄ was only double that of BPA(OH)₁ and/or 2 ($6.0 \pm 0.1 \times 10^{-1}$ M ($n = 1$), $(6.6 \pm 0.1) \times 10^{-1}$ M ($n = 2$), and 1.20 ± 0.05 M ($n = 4$) (11), the $1/D_{37}$ value and/or the uptake of BPA(OH)₄ significantly decreased, probably because the chemical conformation of BPA(OH)₄ itself affects the uptake into tumor cells. These values suggested that they hydroxylform of *dl-p*-BPA(OH)₂ was optimal.

The N:B ratios determined by PGS are shown in Table 1. The boron-10 distribution in normal parenchyma decreased according to the number of -OH groups. The T:N ratios determined by ATA increased in the hydroxylform of BPA, as shown in Table 1. The T:N and/or N:B ratios of BPA(OH)₂ were clearly superior for BPA based BNCT. The T:B ratios (obtained by multiplying T:N by N:B) are also shown in Table 1, and these values were not negatively affected by the hydroxylation of BPA. Although BPA(OH)₁ and 2 showed a higher tumor cell killing effect than BPA, the T:B ratios of BPA(OH)₁ and 2 were not so high. The reasons for this remain unclear. The T:B ratio of the *l*-form was higher than the racemic form of BPA, being similar to that of published studies (4, 5). The *l*-form of BPA(OH)₂ should also be investigated for clinical application. However, the clinical application of the *l*-form or racemic form must be determined by considering their T:N and/or N:B ratios, since the N:B ratio of the

l-form might increase radiation necrosis. The racemic form can be administered at higher doses, for economical reasons, to achieve the higher absolute ^{10}B concentration in tumors. The ^{10}B concentration in the blood in the hydroxylforms of BPA decreased according to the number of -OH groups under our experimental conditions. This reasons for this remains unclear. Hydroxylforms of BPA may be rapidly excreted via the urinary system or may be difficult to absorb by the digestive system after *p.o.* administration. A pharmacokinetic study must determine the most effective way to administer BPA(OH)₂.

The surviving fraction of melanoma cells on BNCT is shown in Fig. 3. The D_{37} values were 0.97×10^{12} nvt for BPA(OH)₂ and 1.44×10^{12} nvt for BPA. The relative tumor cell killing effect was 1.48. The D_{37} values of BPA(OH)₂ and BPA on melanoma cells were both smaller than that of T98G. This means that BPA and BPA(OH)₂ might accumulate in the melanoma cells partially as a specific amino acid due to their metabolic activity. However, no evidence of accumulation of ^{10}B delivered by BPA into melanin by melanogenesis in melanoma has been reported. Yoshino *et al.* (16) have suggested that

Table 1 Ratios of T:N, N:B, and T:B for hydroxylforms of BPA determined by ATA and/or PGS on 9L gliosarcoma-bearing rats^a

	<i>dl</i> -BPA	<i>dl</i> -BPA(OH) ₁	<i>dl</i> -BPA(OH) ₂	<i>l</i> -BPA
T:N ^b	3.5 ± 0.7^c	4.2 ± 0.9	5.0 ± 1.1	3.2 ± 0.7
N:B	0.64 ± 0.16	0.51 ± 0.13	0.43 ± 0.35	0.79 ± 0.09
T:B	2.2 ± 0.73	2.1 ± 0.72	2.2 ± 1.85	2.5 ± 0.63
Blood ppm ¹⁰ B	5.8 ± 0.4	4.5 ± 0.6	2.8 ± 0.8	5.8 ± 0.3
Brain ppm ¹⁰ B	3.7 ± 0.9	2.3 ± 0.5	1.2 ± 0.8	4.6 ± 0.5
Liver ppm ¹⁰ B	7.0 ± 0.5	4.6 ± 0.5	1.8 ± 0.7	5.9 ± 0.8

^a The detectable limit of our PGS was below 1 ppm.

^b Determined by ATA; other parameters and ^{10}B concentration were determined by PGS. The T:B ratios were calculated by multiplying the relating ratios of T:N and N:B.

^c Values are mean \pm SE ($n = 5$).

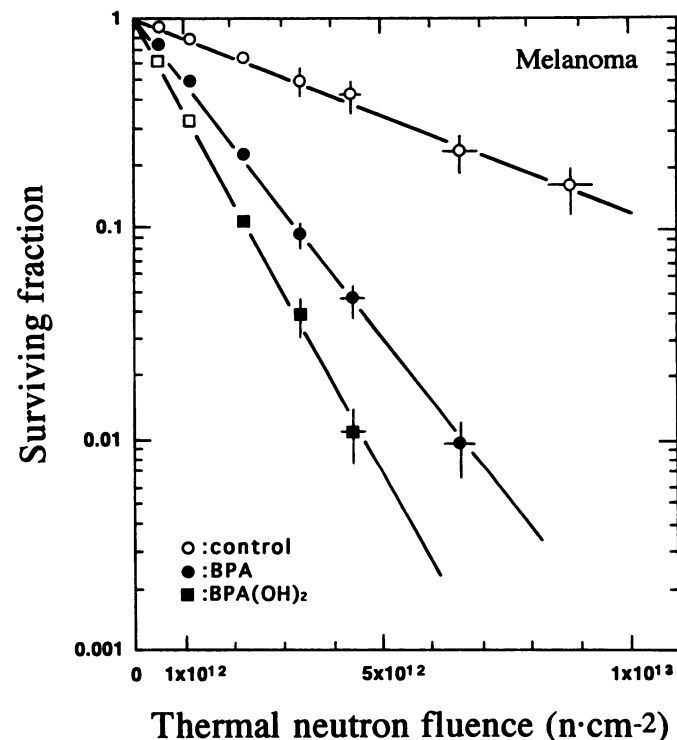


Fig. 3. The surviving fraction of Greene's melanotic melanoma cells after *in vitro* BNCT. Values are means \pm SE.

^{10}B atoms can be removed from melanogenesis by enzymatic activity, although BPA is initiated by tyrosinase.

The water solubility and the cytotoxicity might be irrelevant for clinical use. The water solubility of $\text{BPA}(\text{OH})_2$ was two orders of magnitude higher than that of BPA, being $(6.6 \pm 0.1) \times 10^{-1}$ M and $(7.9 \pm 0.1) \times 10^{-3}$ M, respectively. The IC_{50} value of $\text{BPA}(\text{OH})_2$, defined as the dose that failed to inhibit tumor growth by more than 50% after a 3-day incubation, was nearly one half that of BPA on B-16 melanoma cells: 1.8×10^{-2} M for $\text{BPA}(\text{OH})_2$ and 8.6×10^{-3} M for BPA (11). A fructose complex of BPA is another water-solubilizing form (17). However, it is considered that the fructose complex is immediately converted to BPA by hydrolysis in blood (11).

For BPA-based BNCT, it is desirable to reduce the dose to the normal brain, since the dose distribution ratio of the normal brain parenchyma to the capillary endothelium is nearly 1 (18), which is almost double that of BSH-based BNCT (19). The reduction rate of the absorbed dose on normal parenchyma using $\text{BPA}(\text{OH})_2$ instead of BPA was roughly estimated using the equation $(N_{\text{BPA}(\text{OH})_2}\sigma_{\alpha} + N_{\text{N}}\sigma_{\text{N}})/(N_{\text{BPA}}\sigma_{\alpha} + N_{\text{N}}\sigma_{\text{N}})$, where N is the concentration of ^{10}B and/or ^{14}N in the normal brain and σ is the thermal neutron cross-section of ^{10}B and/or ^{14}N . The RBE values were supposed to be equal for α -rays and protons emitted from $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ and $^{14}\text{N}(\text{n},\text{p})^{14}\text{C}$, respectively, in this calculation. The contribution of core γ -rays is eliminated. Supposing 20 ppm ^{10}B is accumulating in the blood, the rate is 0.79. Therefore, the dose to the normal brain can be reduced about 21% by using $\text{BPA}(\text{OH})_2$ instead of BPA, without a decrease in the selective tumor killing effect and radiation damage to the capillary endothelium. In our preliminary study of BNCT for patients with malignant brain tumors using a *l-p*-BPA-fructose complex, intraoperative BNCT must be used to reduce the dose to the related normal brain surrounding the tumor after tumor debulking. This allowed a therapeutic dose to be absorbed even by the tumor bottom, using a large tumor cavity filled with voids as a neutron penetrator. Despite these surgical maneuvers, early necrosis and general convulsion (rarely status epilepticus) frequently occurred around 3 days after BNCT, since the protection of normal cortex from the radiation injury had to be sometimes sacrificed to fulfill the minimum requirement for the curable dose onto the bottom of the tumor. We believe that the dose to the normal brain and/or capillary endothelium must be minimized to reduce radiation injury to the surrounding normal parenchyma. The hydroxylform of $\text{BPA}(\text{OH})_2$ might be suitable on BPA-based BNCT on malignant brain tumors, since it has lower cytotoxicity and higher water solubility.

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