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Selective Delivery of Boron by the Melanin Precursor Analogue *p*-Boronophenylalanine to Tumors Other Than Melanoma[1]

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ABSTRACT

The melanin precursor analogue *p*-boronophenylalanine (BPA) has been used to deliver ^{10}B to melanoma tissue for boron neutron capture therapy. Uptake studies in tumor models other than melanoma now indicate that BPA is capable of delivering therapeutic amounts of boron to tumors other than melanoma. The KHJJ murine mammary tumor carried s.c. in BALB/c mice, the GS-9L rat glioma carried both s.c. and intracranially in F-344 rats, and the human U-87 MG glioma xenograft carried s.c. in nude mice have all shown significant accumulation of boron in tumor tissue following single p.o. (intragastric) doses of BPA. In the KHJJ mammary tumor, the L isomer of BPA was preferentially accumulated compared to the D isomer, indicative of a carrier-mediated transport process. Double-label, whole-body autoradiographic studies in a pigmented murine melanoma have shown that the boron distribution (from BPA) differs from the distribution of a tritiated melanin precursor (tyrosine). Boron accumulated only in the tumor; labeled tyrosine accumulated in tumor, liver, intestinal epithelium, bone marrow, and secretory glands. Toxicity studies in mice and rabbits indicate that, even at very high doses, BPA p.o. caused no adverse effect in tissues, on blood chemistry, or on differential leukocyte counts. These data indicate that BPA may be generally useful as a boron delivery agent for boron neutron capture therapy of tumors.

INTRODUCTION

Boron neutron capture therapy combines external radiation with tumor-seeking, boron-containing drugs to achieve a selective irradiation of tumor tissue. The densely ionizing heavy charged particles emitted during the $^{10}B(n,\alpha)^{7}Li$ reaction (1) have a range of approximately 5–9 μm in tissue and are known to have a high relative biological effectiveness (2). Selective localization of ^{10}B within the tumor followed by irradiation of the tumor region with low-energy neutrons should allow most of the absorbed dose to be restricted to the tumor. BNCT[3] is dependent on the absolute amount and location of boron in the tumor as well as the tumor/blood and tumor/normal tissue boron concentration ratios. Effective BNCT requires the delivery of considerable amounts (>15 ppm) of ^{10}B into the targeted malignant tissue (3). Difficulties may arise when the concentration of boron in the blood or surrounding normal tissue is equal to or greater than that in the tumor.

When a metabolic difference between tumor and normal tissue can be identified, a rational biochemical approach to loading the tumor with boron becomes possible. In the case of melanoma, advantage can be taken of the biochemical pathways responsible for the production of the pigment melanin. Melanin is synthesized *in vivo* from the aromatic amino acids phenylalanine and tyrosine; melanoma cells actively incorporate these precursors from extracellular fluid. The boron-containing amino acid analogue, BPA (4, 5) has been shown to selectively deliver boron to melanoma tissue (6, 7). BPA has been proved to be effective in therapy neutron irradiations in animal tumor models (8, 9) and has recently been used in Japan in clinical trials for BNCT of human malignant melanoma (10). During our investigations of BPA for BNCT of experimental melanoma, it was noticed that BPA could selectively accumulate in tumors other than melanoma, which is a significant finding considering the dearth of effective delivery agents for BNCT. Results are presented below for BPA distribution studies in a murine mammary adenocarcinoma, in a rat glioma, and in a xenografted human glioma.

MATERIALS AND METHODS

BPA was prepared according to the procedure of Snyder *et al.* (4), with minor modifications (7). The synthesis of BPA utilized ^{10}B-enriched boric acid (95% ^{10}B; Eagle-Picher Industries, Miami, OK); all data presented below were obtained with the ^{10}B-enriched L isomer of BPA. The D and L stereoisomers of BPA were separated by enzymatic resolution of the BPA ethyl esters (5). BPA was administered to animals p.o. (i.g.) as an aqueous slurry by transesophageal intubation. Mice received 15 mg of BPA in 0.5 ml of water; rats received 150 mg of BPA in 3 ml of water; rabbits received 170 mg of BPA in 15 ml of water. [3,5-^{3}H]Tyrosine (specific activity, 42 Ci/mmol) was obtained from ICN Radiochemicals, Irvine, CA.

The analysis of ^{10}B in tissue samples was carried out by prompt-γ ray spectroscopy (11). Whole-body distributions of boron were evaluated by NCR, as described previously (7, 12). Briefly, a solid state track detector was placed in direct contact with a freeze-dried whole-body section, irradiated with thermal neutrons, and chemically etched to produce holes in the emulsion along latent α and ^{7}Li tracks. In NCR images, light areas correspond to areas of high boron concentration. Tritium autoradiography in whole-body sections was performed using Ultrofilm ^{3}H (Pharmacia, Uppsala, Sweden) and exposure times of about 8 weeks. Double-label, whole-body autoradiographic studies utilizing boron (from BPA) and tritium were performed as described by Fand *et al.* (13).

The KHJJ tumor line was derived from a primary mammary tumor that originated in a BALB/c mouse after implantation of a hyperplastic alveolar nodule (14). This tumor line was provided to us by Dr. S. Rockwell, Yale University, and was maintained by serial passage in BALB/c mice. The GS-9L rat glioma cell line was derived from a *N*-nitrosomethylurea-induced tumor (15). Solid tumors were produced s.c. in F-344 rats by the injection of 5×10^{6} cells in 0.1 ml of growth medium. Tumors were palpable 4–5 days after inoculation and had a volume-doubling time of approximately 4 days. For the induction of brain tumors with the GS-9L cell line, 10^{4} cells in 1 μl of culture medium were injected into the right frontal lobe (16). Using aseptic techniques the scalp was incised and a 0.5-mm burr hole made in the skull at a point 3 mm to the left of the midline and 1 mm anterior to the coronal suture. The cells were injected to a depth of 3–4 mm using

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[3] The abbreviations used are: BNCT, boron neutron capture therapy; BPA, *p*-boronophenylalanine; ^{10}B-L-BPA, ^{10}B-enriched (95 atom %) L stereoisomer of BPA; BSH, $Na_2B_{12}H_{11}SH$; BSSB, dimer of BSH, $Na_4B_{24}H_{22}S_2$; i.g., intragastrically; i.c., intracranially; NCR, neutron capture radiography.

a 27-gauge needle fitted within a Teflon collar. Rats with implanted brain tumors survived for an average of 22 ± 4 (SD) days. The U-87 MG human glioblastoma cell line (17) was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum. Solid tumors were produced in athymic mice (*nu/nu* genotype, NIH Swiss background) following the s.c. injection of 2.5×10^6 cells in 50 μl of culture medium. Tumors weighed about 500 mg after 4 weeks of growth.

RESULTS AND DISCUSSION

The use of BPA as an effective boron-carrying agent for BNCT of experimental melanoma has been reported by Hatta *et al.* (6); BNCT clinical trials have recently been initiated in Japan with BPA for treatment of malignant melanoma (10). We have also been engaged in the development of BPA for BNCT of melanoma (7, 9). In previous reports, we stated that, based on preliminary data with small numbers of mice, BPA did not appear to selectively accumulate in two control (non-melanoma) tumors, a murine mammary adenocarcinoma (7) and the KHJJ murine mammary tumor (9). Improvements in the mode of BPA administration (p.o. rather than i.p. dosing) have resulted in substantially higher levels of boron in melanoma tissue (9). As a consequence of these higher doses of BPA, we were able to detect a preferential accumulation of boron in the KHJJ tumor. This observation led us to examine more closely the possibility that BPA may have a more general utility as a tumor-seeking drug for BNCT.

Fig. 1 shows the boron concentration in the KHJJ murine mammary tumor and normal tissues as a function of time following the p.o. administration of 15 mg of either the L isomer of BPA (Fig. 1*A*) or the D isomer (Fig. 1*B*). The boron concentration in tumor was significantly higher with the L isomer than with the D isomer. This would be expected if the accumulation within the tumor were a carrier-mediated transport process. We have previously reported that the L isomer of BPA showed preferential uptake (compared to the D isomer) in the Harding-Passey melanoma (7). In most BPA biodistribution studies, we have noted that boron concentrations in blood and in other normal tissues are similar and follow the same clearance time course. It is interesting to note that, as was seen in tumor, the blood and normal tissue boron concentrations are somewhat higher following administration of L-BPA than of D-BPA. This is consistent with our assumption that BPA is passing as an amino acid analogue; the L isomer enters metabolic pathways but the D isomer does not. The boron concentration in the KHJJ tumor was within the range considered to be adequate for effective BNCT: >15 ppm (3).

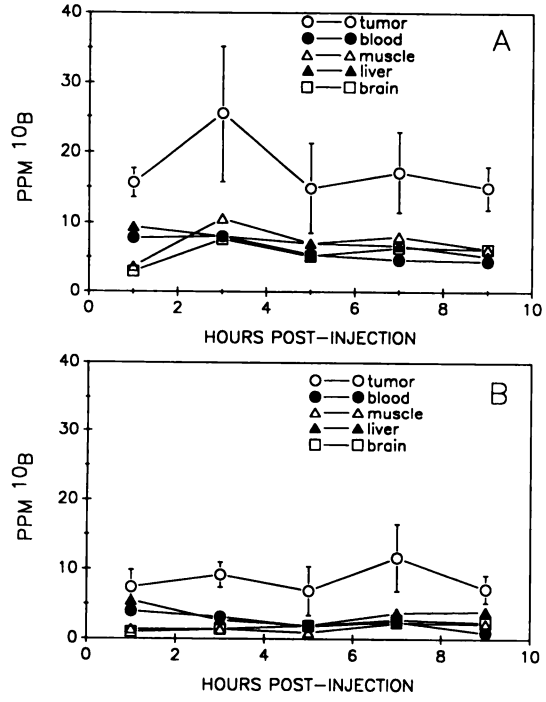

Fig. 1. Boron concentration in tumor and normal tissue in the KHJJ murine mammary tumor. Each *point* is the average of 5 mice [mean ± SD (*bars*)]. Each mouse received 15 mg of the L isomer (*A*) or the D isomer (*B*) of BPA given p.o. as a slurry in 0.5 ml of water.

The GS-9L rat glioma model has been used to evaluate the tumor uptake and biodistribution of other boron-containing compounds for BNCT (16), including the sulfhydryl borane, $Na_2B_{12}H_{11}SH$ or BSH, currently in clinical use in Japan for BNCT of glioblastoma. We have carried out a biodistribution study in this tumor model with BPA; the results are shown in Fig. 2. The time course of boron uptake in the glioma shows a broad maximum at 5–9 h after the p.o. administration of 150 mg L-BPA. During this time the boron concentration in the tumor is within the therapeutic range, and the tumor/blood boron concentration ratio is in the range between 3/1 and 4/1. The relatively late and broad time window for achieving the maximum amount of boron in the tumor admits the possibility of using a multiple dosing regimen to boost the tumor concentration. These experiments are in progress. BPA was also administered to 4 rats with i.c. GS-9L gliomas. Two were sacrificed at 5 h and 2 at 7 h postadministration. The boron concentrations in tumor and blood for these 4 animals fell precisely within the error bars shown in Fig. 2 (5 h: tumor, 15.2, 21.8; blood, 3.7, 6.5; 7 h: tumor, 19.3, 22.9; blood, 6.5, 7.0).

In clinical application, BSH produces tumor/blood boron concentration ratios of approximately 1.5 (18). The sulfhydryl boranes (BSH and BSSB) must be given slowly as continuous infusions for maximum tumor boron loading (19). The pharmacokinetic studies of Joel *et al.* (16) have shown that the dimer form of BSH, BSSB, may be superior to BSH with respect to retention in the tumor following termination of the infusion. The high levels of boron in the blood following infusions of these boranes is a potential problem. Major pharmacological manipulations such as blood transfusions or plasmapheresis are required to produce tumor/blood ratios of about 3 while maintaining therapeutic levels of boron in the tumor (16). In contrast, a single p.o. dose of BPA has produced the same end result in the GS-9L rat glioma model (see Fig. 2); *i.e.*,

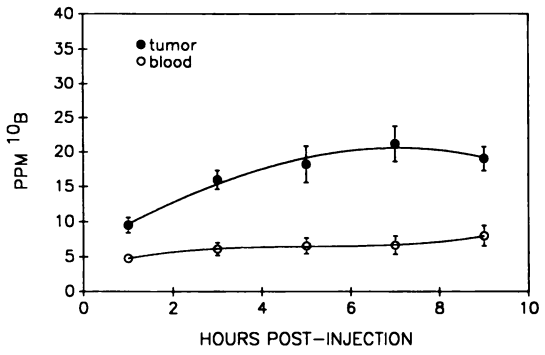

Fig. 2. Boron concentrations in tumor and blood in the GS-9L rat glioma carried in the Fischer 344 rat. Ten rats were implanted with five s.c. tumors each; one tumor and a sample of blood was taken from each rat at the indicated time points. Data *points* (n = 10) are mean ± SD (*bars*). Each rat received 150 mg of ^{10}B-L-BPA p.o. as a slurry in 3 ml of water.

therapeutic levels of boron in the tumor with a tumor/blood ratio of 4. Table 1 gives a comparison of tumor (glioma) and blood boron concentration values for BPA, BSH, and BSSB in the GS-9L rat glioma model; the data for BSH and BSSB are taken from the report of Joel *et al.* (16). The question must be raised as to whether BPA is a more effective boron delivery agent for glioma than the sulfhydryl boranes. Double-label experiments with BPA and tritiated thymidine for comparison of the boron distribution within the tumor to the tritium distribution in the same whole-body section have shown that BPA selectively delivered boron to those areas of the tumor identified as actively dividing by uptake of tritiated thymidine (7). On the other hand, such a double label experiment using BSH and tritiated thymidine in a pigmented murine melanoma showed that BSH accumulated to a greater degree in the nonproliferating regions of the tumor (20). These compounds (BPA and the sulfhydryl boranes) are most probably localized in tumor by different mechanisms. A combination of BPA and BSH (or BSSB) may provide a more uniform boron distribution within the tumor resulting in more effective BNCT.

Fig. 3 shows the results of a preliminary BPA distribution study carried out in the U-87 MG human glioma xenograft carried s.c. in the nude mouse. The accumulation of boron in the tumor shows a broad maximum but the absolute amount of boron achieved in the human glioma is somewhat less than that observed with the GS-9L rat glioma.

The accumulation of BPA in rapidly growing animal tumors (including melanomas) could be due to the metabolic demand for the amino acids needed for protein synthesis. To address this question, we have initiated studies designed to compare the whole-body distribution of boron (from BPA) with the distribution patterns of labeled amino acids. Fig. 4 shows the results of a double-label study using BPA and tritiated tyrosine in a pigmented B16 melanoma carried in a C57BL/6 mouse. The boron distribution NCR following p.o. administration of BPA

Table 1 *Comparison of BPA and the sulfhydryl boranes in a rat glioma model*

Compound	Blood (ppm ^{10}B)	Tumor (ppm ^{10}B)
BPA[a]	5	20
BSH[b]	27	20
BSSB[b]	48	37
BSSB[c]	10	32

[a] Single p.o. dose of BPA, 35 μg ^{10}B/g body weight; data from Fig. 2.

[b] Boron concentrations immediately following a continuous infusion (i.p. or i.v.) at a rate of 50–60 μg ^{10}B/g body weight/day for 3 or 4 days; data from Ref. 16.

[c] Dimer was administered as in Footnote *b*; immediately following the end of the infusion a 2-blood volume plasmapheresis was carried out in a time span of 2 h; the rats were then sacrificed and boron analysis was carried out; data from Ref. 16.

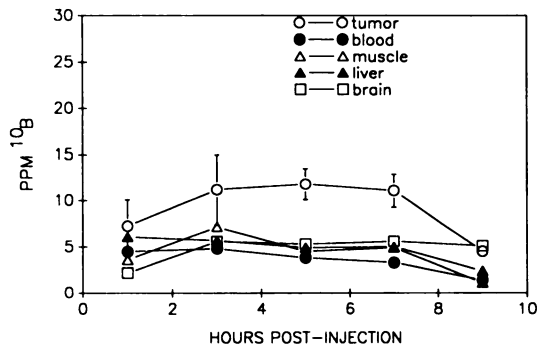

Fig. 3. Boron concentrations in tumor and normal tissues in the U-87 MG human glioma xenograft carried s.c. in the nude mouse. Each mouse received 15 mg ^{10}B-L-BPA p.o. as a slurry in 0.5 ml water. Each mouse carried 2 or 3 tumors; each *point* represents 2 mice (tumor values, n = 4–6). *Bars*, SD.

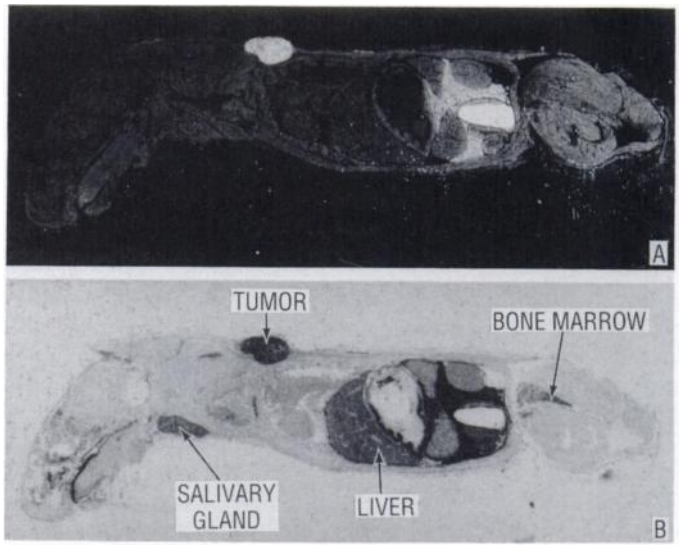

Fig. 4. Double labeling with boron and [3,5-^{3}H]tyrosine. A tumor-bearing mouse was given injections of both ^{10}B-L-BPA (15 mg p.o., 6 h before sacrifice) and [3,5-^{3}H]tyrosine (10 μCi/g body weight i.p., 1 h before sacrifice); whole-body sections were prepared. *A*, NCR prepared from the whole-body section; bright areas correspond to high boron concentrations. *B*, the [3,5-^{3}H]tyrosine autoradiogram prepared from the same whole-body section used for the NCR; dark areas indicate areas of [3,5-^{3}H]tyrosine accumulation (with the exception of the peritoneal cavity, which reflects residual activity from the i.p. injection). Note that the boron is selectively accumulated in the tumor, whereas the [3,5-^{3}H]tyrosine has been distributed to all metabolically active tissues.

(Fig. 4*A*) and the whole-body tritium autoradiogram resulting from the labeled tyrosine (Fig. 4*B*) were prepared from the same whole-body section. Tyrosine, a true melanin precursor, has been incorporated into all metabolically active tissues; the intestinal epithelium, liver, bone marrow in the femur, secretory glands, and the tumor are labeled in Fig. 4*B*. The NCR of the same whole-body section shows that the boron is accumulated primarily in the tumor and not in the other types of rapidly dividing tissues. The fact that the boron distribution (from BPA) does not match the distribution of the melanin precursor tyrosine was unexpected. This potentially important observation will require further study. In Fig. 4*A*, high boron concentration is evident in the abdominal area in a cross-section of intestinal tract and is a result of the p.o. administration route used for BPA.

Our previous distribution studies with BPA indicated that the boron concentration achieved in tumor was proportional to the injected dose of BPA (7). BPA can be given in the large doses needed for effective tumor loading due to its low toxicity. Fifty % lethal dose values have been reported for BPA (21, 22). The extremes of pH utilized to solubilize the BPA prior to i.p. or i.v. injections were, most likely, responsible for the reported toxicity. BPA is least soluble at near-physiological pH (isoelectric point, 6.2); solubility increases significantly at either high or low pH. We have encountered the same problems with (lack of) solubility and have shown that administration of BPA p.o. as an aqueous slurry at neutral pH is an acceptable alternative to i.p. or i.v. injections (9). The time course of boron accumulation in the tumor after p.o. administration was approximately the same as that observed following i.p. injections. Administration p.o. has allowed us to give larger bolus doses with correspondingly higher boron levels in melanoma, higher than was obtained using i.p. injections (40 ppm *versus* 15 ppm in the tumor). Accordingly, we have carried out the following toxicity studies in mice and rabbits using nonenriched DL-BPA administered p.o. as an aqueous slurry.

Acute Study. Six mice/group; BPA group received a single

p.o. dose of 20 mg BPA (1000 mg/kg) in 0.5 ml water; control group received water only; sacrifice at 1 week postinjection.

Chronic Study. Six mice/group; BPA group received one p.o. dose of 10 mg BPA in 0.5 ml water/day, 5 days/week for 2 weeks (total dose, 5000 mg/kg); control group received water on the same schedule; sacrifice 2 and 4 weeks after the final dose.

Chronic Study. Four rabbits/group; BPA group was given 170 mg BPA p.o. in 15 ml water for 5 consecutive days, then 2 days off, then 2 more daily doses (total dose, 525 mg/kg); control group received 15-ml doses of water on the same schedule; sacrifice (2 controls and 2 treated) at 2 weeks and 4 weeks after the final dose.

Histopathology. The following tissues were sent to Experimental Pathology Laboratories, Inc., Sterling, VA. Mice: brain, heart, spleen, liver, kidney, adrenal, lung, stomach, duodenum, jejunum, ileum, cecum, colon; rabbits: adrenal, spleen, duodenum, ileum with Peyer's patch, jejunum, stomach, kidney, liver, uterus, ovary, lung, heart, thymus, brain.

Blood samples were taken from the rabbits during the toxicity study and at the time of sacrifice. The blood analyses were performed at the Clinical Research Center of the Medical Department, Brookhaven National Laboratory.

In summary, the histopathology reports from Experimental Pathology Laboratories indicated that microscopic examination of hematoxylin and eosin-stained tissue sections showed no compound-related alteration in any tissue. The blood clinical chemistries showed no significant variation from control values. Differential leukocyte counts from the chronically dosed rabbits showed no deviation from normal.

We have recently initiated a Phase I distribution study of BPA in patients with malignant melanoma. Efforts are under way in this country to initiate clinical trials of BSH for BNCT of human glioblastoma along the lines of the Japanese BNCT program (18). The preliminary data presented above suggest that BPA is not restricted to melanoma in its ability to selectively deliver boron to malignant tissue and should be considered as an alternative single-agent boron delivery compound for BNCT of glioblastoma. A combination of boron-containing tumor-seeking compounds may be needed in some cases to achieve the maximum possible degree of tumor boron loading. BPA, with its favorable tumor-to-blood distribution characteristics, extremely low toxicity, and ability to deliver boron to a variety of tumors, is one of the more promising compounds developed for BNCT of human neoplasms.

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