Selective Targeting of Boronophenylalanine to Melanoma in BALB/c Mice for Neutron Capture Therapy

Jeffrey A. Codere, John D. Glass, Ralph G. Fairchild, Uma Roy, Scott Cohen, and Irwin Fand

Medical Department, Brookhaven National Laboratory, Upton, New York 11973 [J. A. C., J. D. G., R. G. F.]; Department of Physiology and Biophysics, Mt. Sinai School of Medicine, 5th Avenue at 100th Street, New York, New York 10029 [J. D. G., U. R.]; and Institute of Molecular Immunology, Center for Molecular Medicine and Immunology, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103 [S. C., I. F.]

ABSTRACT

Melanoma cells actively accumulate aromatic amino acids for use as precursors in the synthesis of the pigment melanin. Using the Harding-Passey melanoma carried s.c. in BALB/c mice, we have demonstrated that p-boronophenylalanine (BPA) is taken up by melanoma tissue to a much greater extent than by normal tissues. Following a single i.p. injection, or a series of injections given over 1 h, the accumulation of boron in melanoma was found to be transient, reaching a maximum approximately 6 h postinjection. The concentrations of boron achieved in tumor ranged from 9–33 μg/g, and are within the range estimated to be necessary for successful application of the nuclear reaction 10B(n,α)Li for neutron capture therapy. Boron concentrations in tumor and tissues were determined using either a prompt-gamma spectroscopic technique or by quantitative neutron capture radiography using whole-body sections. Distribution studies with the resolved stereoisomers of BPA indicated that the l isomer is preferentially accumulated in the melanoma compared to the d isomer. The l isomer of BPA was shown to be targeted to actively dividing tumor cells by simultaneously comparing the boron and 3H thyminidine distribution in tumor.

Under conditions which selectively deliver high concentrations of boron to Harding-Passey melanomas in BALB/c mice, BPA did not deliver useful concentrations of boron to a mammary adenocarcinoma in Hale-Stoner mice. These results, along with the selectivity of the Harding-Passey melanoma for the l isomer of BPA, are consistent with our working hypothesis that BPA is actively transported into the melanomas as an analogue of natural melanin precursors.

INTRODUCTION

During conventional radiation therapy, the dose that can be delivered to the tumor is limited by the tolerance of the surrounding normal tissues within the treatment volume. NCT1 represents a promising modality for selective irradiation of tumor tissue. 10B nuclei capture thermal neutrons efficiently (effective capture cross-section, 3838 barns) and immediately undergo a nuclear fission reaction. The heavy charged particles from the 10B(n,α)Li reaction (1) have a range of approximately 10 μm in tissue and are known to have a high relative biological effectiveness (2). Localization of 10B in tumor and the subsequent irradiation of this area with low-energy neutrons allows the dose to adjacent normal tissues to be minimized, while at the same time the neutron capture reaction in tumor can be adjusted to bring the absorbed dose into the therapeutic range.

Past clinical trials of NCT for malignant glioma in the United States, failed. Poor results were attributed to vascular damage due to high boron concentrations in blood (tumor/blood ratios < 1), and inadequate penetration of the incident thermal neutron beam in tissue (3). Clinical studies currently underway in Japan utilize the boron cage compound BSH which does show some selective accumulation in tumor; the ratios of concentrations in tumor and blood are approximately equal to 1.5 (4). Further complications in the latter studies are introduced through the use of a thermal neutron beam of low intensity (reactor power, 100 kW), thus necessitating 3- to 5-h irradiations. Nevertheless, it has been reported that median survival has been extended relative to conventional radiotherapy (4, 5).

Our overall aim is to exploit the full potential of neutron capture therapy by utilizing boron-containing compounds that are selectively localized in tumors, and to use an epithermal neutron beam with improved tissue penetration characteristics that significantly reduce normal tissue doses compared to doses from thermal neutron beams. As a tumor model system we use the Harding-Passey melanoma carried in BALB/c mice and a boron-containing analogue of phenylalanine as the biochemically targeted boron carrier (6). Melanin-producing melanomas actively transport and metabolize aromatic amino acids for use as precursors in the synthesis of the pigment melanin. Distribution studies of BPA in our murine melanoma model show that BPA is selectively accumulated in tumor to levels that are within the range needed for effective NCT (15–30 μg 10B/g tumor; see Ref. 7).

MATERIALS AND METHODS

Preparation of BPA. The published synthetic procedure of Snyder et al. (8) was modified by using an acid-catalyzed rather than a base-catalyzed hydrolysis to convert the N-acetyl-p-boronophenylalanine to p-borono-DL-phenylalanine. One g of N-acetyl-p-boronophenylalanine was dissolved in 40 ml of 2 M HCl and refluxed for 2 h. The reaction mixture was evaporated to dryness, followed by the evaporation of distilled water several times to remove traces of HCl. The residue (p-borono-DL-phenylalanine hydrochloride) was dissolved in 15 ml distilled water and the pH was adjusted to 6.2 with NH₄OH to form a white precipitate. The solution was cooled to 4°C and allowed to crystallize overnight. The crystalline product was collected by filtration and recrystallized from water to yield 657 mg (79%) of p-borono-DL-phenylalanine.

Preparation of 10B-enriched BPA. 10B-enriched boric acid (95% 10B; Eagle-Picher Industries, Inc., Miami, OK) was converted to the tri-n-butyl ester by repeatedly evaporating n-butyl alcohol from boric acid with azotropic removal of water until the esterification is complete (9). The synthesis then proceeded as described (8), with the modification noted above.

Resolution of the Stereoisomers of BPA. p-Borono-D-L-phenylalanine was prepared essentially according to the method of Roberts et al. (10), using a general strategy for enzymatic resolution of substituted phenylalanine derivatives originally described by Tong et al. (11). Approximately 1.2 g of p-borono-DL-phenylalanine ethyl ester hydrochloride was dissolved in 30 ml distilled water. The pH of the solution was adjusted to 5.0 with 0.2 M LiOH. α-Chymotrypsin (200 mg; Sigma Chemical Co.; No. C-4129) was added and the mixture was incubated at room temperature. The pH of the solution was maintained at 5 by the addition of 0.2 M LiOH. During the enzymatic reaction the...
solubility limit of \( p \)-borono-L-phenylalanine was exceeded and the product began to crystallize from the reaction mixture. After 3–4 h, the hydrolysis was complete and base was no longer being consumed to maintain the pH at 5. The pH of the reaction mixture was brought to 6.2 with 0.2 M LiOH. Ethanol (6 ml) was added and the mixture was cooled to 4°C and left overnight. The white, crystalline product was collected by filtration and recrystallized from 75 ml of distilled water. The crystalline \( p \)-borono-L-phenylalanine produced in this way was chromatographically identical (thin layer and ion exchange chromatography) to the racemic material and to the \( l \) isomer prepared by the action of renal acylase on \( N \)-acyetyl-\( p \)-borono-DL-phenylalanine (12). The reaction has been scaled up to process as much as 3.4 g of the racemic amino acid ester with no problems. The yields of the \( l \) isomer have averaged about 75% of the theoretical amount.

Residual \( p \)-borono-D-phenylalanine ethyl ester was recovered from the chymotryptic digestion mixture by extraction of the aqueous solution with ethyl acetate by pH 9. The ethyl acetate was removed by rotary evaporation and the ester was treated with refluxing 2 M HCl for 2 h. The aqueous acid was removed by rotary evaporation, the residual \( p \)-borono-D-phenylalanine hydrochloride was taken up in a minimal amount of water, and the pH of the solution was adjusted to about 6.8. The \( d \) amino acid began to crystallize immediately. After 2 days at 4°C the product was collected by filtration, washed with cold water, and dried in vacuo.

Murine Melanoma Model. The experiments described below were carried out in adult female BALB/c mice in which the Harding-Passey melanoma was implanted s.c. This model has been found to be quite reproducible with respect to melanin content and uptake of melanin-affinic agents (13). Further, melanin content (0.68% by weight melanin) is similar to that found in human melanotic melanoma (0.1 to 0.8%; average value, 0.35%) (14). Each experiment incorporates data from the same or adjacent tumor passages. Mice weighed about 20 g and were approximately 10 weeks old. Pieces of minced tumor (1–2 mg) were implanted by trocar s.c. on the abdomen or on the thigh. No difference in drug uptake has been observed for abdomen or thigh tumors following i.p. injection of several different drugs.

Biodistribution Studies. Solutions for injection were prepared by dissolving the BPA in 0.1 M Tris-HCl, pH 8.0, and sterilized by passage through a 0.22-\( \mu \)m filter. Injections were given i.p. under light ether anesthesia. Mice were killed by ether inhalation and their tissues were analyzed for boron content as described below. Tissues were weighed immediately after dissection and are thus designated “wet weights.”

Results are expressed as \( \mu \)g boron per g tissue.

Boron Gamma-Boron Analysis. A prompt-gamma boron analysis technique is used in which the 478 keV \( \gamma \)-photons produced during the \( ^{10}\text{B}(n,\gamma)^{11}\text{Li} \) reaction are measured to determine boron content in tissue samples weighing up to 1.0 g. The details of the prompt-gamma boron analysis facility have been described (15). Determination of \( \mu \)g amounts of \( ^{10}\text{B} \) in tissue can be made in 200 s with an error (± 1 SD) of ~ 15%.

Fig. 1. Tumor and normal tissue distribution of \(^{10}\text{B}-\text{L-BPA}. \) Following a single i.p. injection, the boron concentration in tumor exceeds that in normal tissues by a factor of 3–10. The uptake of boron by the tumor is transient, reaching a maximum at 6 h postinjection and returning to background levels by 24 h postinjection.

There were considerable variations in tumor levels of BPA from one experiment to the next. These fluctuations could be accounted for by factors such as the mode of drug administration and, in some experiments, by the presence of residual \( ^{10}\text{B} \) in the tumor. A continuous i.v. infusion did not result in the gradual buildup of BPA in the tumor. This implies that BPA is transported into the melanoma cells but that the boron is not incorporated into the melanin pigment.

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**RESULTS**

Biodistribution of BPA. Fig. 1 shows the results of a biodistribution experiment using \(^{10}\text{B}-\text{L-BPA}. \) Mice (4/group) were each given a single i.p. injection of \(^{10}\text{B}-\text{L-BPA} \) (1.0 ml; 5 mg of \(^{10}\text{B}-\text{L-BPA} \) in 0.1 M Tris, pH 8) and sacrificed at the indicated times. Tissues were analyzed for boron content by the prompt-gamma method. The levels of \(^{10}\text{B} \) in tumor are significantly higher than in other tissues. The boron content of the tumor reaches a maximum 6 h postinjection and then declines to background levels by 24 h postinjection.

Table 1 shows biodistribution experiments with \(^{10}\text{B}-\text{L-BPA} \) that examine some effects of variation in the mode of drug administration. These data confirm the transient nature of the BPA accumulation in the melanoma. A continuous i.v. infusion did not result in the gradual buildup of BPA in the tumor. This implies that BPA is transported into the melanoma cells but that the boron is not incorporated into the melanin pigment.
Female BALB/c mice carrying the Harding-Passey melanoma were given $^{10}$B-L-BPA by various modes of administration. The mice were sacrificed at the times indicated and freshly dissected tissue samples were analyzed for boron content by the prompt-gamma method. Values are the mean ± SD.

### Table 1 Biodistribution data for $^{10}$B-p-borono-L-phenylalanine

<table>
<thead>
<tr>
<th>Time of sacrifice (h)</th>
<th>Thigh tumors</th>
<th>Tumor</th>
<th>Blood</th>
<th>Tumor/blood ratio</th>
<th>Tumor/muscle ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (n = 3)</td>
<td>14.5 ± 6.0</td>
<td>4.2 ± 0.7</td>
<td>5.1 ± 2.1</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>6 (n = 6)</td>
<td>12.6 ± 5.1</td>
<td>1.7 ± 1.4</td>
<td>3.6 ± 0.9</td>
<td>7.4</td>
<td>3.5</td>
</tr>
<tr>
<td>8 (n = 3)</td>
<td>8.3 ± 3.1</td>
<td>1.1 ± 0.4</td>
<td>2.8 ± 0.8</td>
<td>7.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Abdominal tumors*</td>
<td>6 (n = 3)</td>
<td>18.6 ± 12</td>
<td>3.4 ± 0.6</td>
<td>5.2 ± 1.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Abdominal tumors†</td>
<td>6 (n = 12)</td>
<td>8.8 ± 2.8</td>
<td>1.7 ± 1.3</td>
<td>3.2 ± 0.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Abdominal tumors‡</td>
<td>12 (n = 3)</td>
<td>8 ± 3</td>
<td>ND</td>
<td>1.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Thigh tumors‡</td>
<td>6 (n = 5)</td>
<td>11 ± 2</td>
<td>0.9 ± 0.4</td>
<td>1.9 ± 1.2</td>
<td>12</td>
</tr>
</tbody>
</table>

* Mice received a single i.p. injection of 6 mg BPA in 1.0 ml 0.1 M Tris, pH 8.0.
† Mice received 2 i.p. injections 45 min apart, each injection contained 6 mg of BPA in 1.0 ml 0.1 M Tris, pH 8.0. The mice were sacrificed 6 h after the second injection.
‡ Mice received 26 mg BPA during a 78-h continuous infusion into the tail vein at a rate of 2 ml/day and were sacrificed 12 h after the end of the infusion.

Uptake of BPA was a Function of Injected Dose. The solubility limit of BPA at about neutral pH is approximately 12 mg/ml. To determine whether lower doses would give comparable levels of tumor loading and less background loading of normal tissues, the experiment summarized in Fig. 2 was carried out. Malignant-bearing mice (3/point) were given injections (i.p., 1.0 ml) of solutions of $^{10}$B-L-BPA in 0.1 M Tris, pH 8.0, at concentrations of 3, 6, and 12 mg/ml. The mice were sacrificed 3 h postinjection and the tissues were analyzed for boron content by the prompt-gamma method. The data in Fig. 2 show that normal tissues show no obvious dose response. The boron content of tumor, however, increases markedly with injected dose.

**Uptake of BPA in a Mammary Carcinoma.** It is assumed that the selective accumulation of BPA in melanoma is somehow linked to melanin biosynthesis. To test this assumption and to ascertain whether BPA may have a more general usefulness as a tumor-localizing agent, an uptake study was performed using an adenocarcinoma of the breast carried in Hale-Stoner mice (courtesy of Dr. E. P. Cronkite, Medical Department, Brookhaven National Laboratory). The results are shown in Table 2. It can be seen that the tumor level of boron reaches a maximum of approximately 6.5 $\mu$g $^{10}$B/g tumor 3 h after i.p. injection and drops to below the limit of detection by 12 h. The uptake and clearance of BPA from the adenocarcinoma are similar to those observed in normal tissues. Under the same conditions boron levels at 3 h postinjection in our murine melanoma are in excess of 20 $\mu$g $^{10}$B/g tumor (see Fig. 2). The concentrations of boron measured in the adenocarcinoma tumor are too low to be useful in neutron capture therapy.

### Neutron Capture Radiography

Fig. 3 shows a whole-body section (A) and the corresponding neutron capture radiogram (B) from a melanoma-bearing mouse that had been given an injection of 12 mg of $^{10}$B-L-BPA (6 mg/ml in 0.1 M Tris, pH 8.0; four 0.5-ml i.p. injections over the course of 1 h). The mouse was sacrificed and frozen 6 h after the final injection. Boron-containing standards are visible above the whole-body section. The standards were prepared with boric acid containing natural abundance boron (i.e., 20% $^{10}$B) and, from left to right, 6.5 ± 0.6 2.8 ± 0.8 1.8 ± 0.6

<table>
<thead>
<tr>
<th>Time of sacrifice (h)</th>
<th>Tumor</th>
<th>Blood</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>3*</td>
<td>6.5 ± 2.8</td>
<td>2.7 ± 0.6</td>
<td>ND</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>6*</td>
<td>2.7 ± 1.2</td>
<td>0.3 ± 0.4</td>
<td>ND</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>12*</td>
<td>ND</td>
<td>1.8 ± 0.6</td>
<td>ND</td>
<td>2.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Hale-Stoner mice (4/point) carrying a mammary adenocarcinoma transplanted s.c. on the back were given injections i.p. of 1.0 ml of $^{10}$B-L-BPA (6 mg/ml in 0.1 M Tris buffer, pH 8.0), and were sacrificed at the indicated times. Tumor and blood samples were analyzed for boron content by the prompt-gamma technique. Values are the mean ± SD.
† ND, not detectable.
‡ Mice (3/point), treated as described above, were frozen and whole-body sections were prepared. Neutron capture radiograms were prepared from the whole-body sections. Boron concentrations were determined by comparison to boron-containing standards processed simultaneously during the slicing and irradiation procedures.
correspond to 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μg B/g tissue. It is readily apparent that the tumor contains high levels of boron. There are also areas within the abdominal cavity which contain high levels of boron, most probably the stomach, intestines, and pancreas, as well as some possible local adsorption artifact resulting from the multiple i.p. injections. The boron distribution across the relatively large tumor shown in Fig. 3 is nonuniform; the boron concentrations ranged from 9.3 to 21 μg 10B/g and averaged 14 μg/g. Boron concentrations obtained from the neutron capture radiogram shown in Fig. 3 are similar to values obtained by prompt-gamma boron analyses. Results (μg 10B/g) for Fig. 3: tumor, 14; liver, 3.0; muscle, 2.8; brain, 4.8; pancreas, 17; lung, 3.0; blood, 2.9; skin, 2.0; tumor/blood ratio, 4.8; tumor/muscle ratio, 5.0.

Fig. 4 shows the results of an experiment in which tumor-bearing mice were given injections (i.p.) of both 10B-L-BPA (6 mg in 1.0 ml 0.1 M Tris, pH 8, 6 h before sacrifice) and [9H]-Thd (10 μCi/g body weight, 2 h before sacrifice) to determine whether the boron is being delivered to viable areas of tumor. The large tumor used for Fig. 4 was chosen in order to contrast the radiation technique has been used here to compare the viable and replicating tissue labeled with [3H]Thd. The distribution of boron within the tumor precisely matches the area of viable and replicating tissue labeled with [3H]Thd.

**DISCUSSION**

BPA was synthesized by Snyder et al. (8) nearly 30 years ago with the idea that it should be useful for neutron capture therapy. Early studies with BPA concentrated on the concept of tumor isolation by the blood-brain barrier (20). More recently investigators in Japan have taken some note of the status of the compound as an aromatic amino acid analogue by studying its loading of boron into melanoma cells. Ichihashi et al. (21, 22) and Itsumi et al. (23) have described the enhanced killing effect of thermal neutrons on melanoma cells in vitro that had been preincubated with 10B-enriched BPA. Mishima et al. (24) have also described the use of BPA to effect an apparent cure of a spontaneously occurring melanoma in a Duroc pig, and to suppress the growth of the Greene melanoma in hamsters following a single thermal neutron treatment (24, 25). In the pig experiment the BPA was injected topically around the lesion immediately before the irradiation. The amount of boron incorporated into the hamster melanoma ranged from 3 to 12 μg boron/g tissue which is considerably lower than the 30 μg 10B/g tissue estimated to be necessary for effective NCT using a thermal neutron beam (7). All of the work cited above was done with the racemic amino acid.

Our preliminary animal experiments with racemic 10B-enriched BPA indicated that the amino acid was concentrated by tumor tissue and that the highest differential concentrations of boron were obtained at relatively short time intervals after injection of the amino acid. Under these conditions it was considered advantageous to resolve the stereoisomers and work with the pure 10B-L-BPA without background interference from the D isomer. Further studies are under way in our laboratory to evaluate the pharmacokinetics of the purified D isomer.

The results described above demonstrate that BPA is actively accumulated by melanoma tissue to a greater degree than by normal tissues, and complement data reported by others (24–26). The BPA buildup in tumor is transient, reaching maximum levels approximately 6 h postinjection. With other compounds, a continuous i.p. or i.v. infusion results in the gradual buildup of drug in the murine melanoma (27). However, this is not the case with BPA. A long-term i.v. infusion produced tumor levels of boron not significantly different from those obtained following single injections. These data indicate that boron is not being incorporated into melanin. Whether borate has been oxidatively cleaved from the amino acid or whether the BPA is excreted intact remains to be determined.

The uptake of BPA in a nonmelanotic tumor was measured in order to test our assumption that the accumulation of BPA in melanoma tissue is due to the increased rate of transport and metabolism of aromatic amino acids for the production of melanin. The results show that, for at least an adenocarcinoma,
there is no therapeutically significant concentration of BPA by the tumor as compared to normal tissues. Interestingly, preliminary results with a poorly pigmented B-16 murine melanoma indicate that BPA is still selectively accumulated in the tumor, even though the melanin content of the tumor is very low. These studies are being pursued in our laboratory and could have significant implications for NCT of malignant melanoma. Additional support for a metabolic mechanism for the selective uptake of BPA in melanoma tissue comes from our observations that the physiological L isomer is accumulated to a much greater extent than the D isomer.

Following i.p. injection of BPA, Harding-Passey melanoma levels of boron are within the range estimated to be necessary for effective neutron capture therapy (7). However the ratio of boron content in tumor to that in normal tissue, especially blood, is also of critical importance in neutron capture therapy. Our results show that BPA exhibits a selectivity for melanoma and a slower rate of clearance from tumor than from normal
ACKNOWLEDGMENTS

The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

REFERENCES


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