The Hamster Cheek Pouch as a Model of Oral Cancer for Boron Neutron Capture Therapy Studies: Selective Delivery of Boron by Boronophenylalanine

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ABSTRACT

Herein we propose and validate the hamster cheek pouch model of oral cancer for boron neutron capture therapy (BNCT) studies. This model serves to explore new applications of the technique, study the biology and radiobiology of BNCT, and assess the uptake of boron compounds and response of tumor, precancerous tissue, and clinically relevant normal tissues. These issues are central to evaluating and improving the therapeutic gain of BNCT. The success of BNCT is dependent on the absolute amount of boron in the tumor, and the tumor:blood and tumor:normal tissue boron concentration ratios. Within this context, biodistribution studies are pivotal. Tumors were induced in the hamsters with a carcinogenesis protocol that uses dimethyl-1,2-benzanthracene and mimics spontaneous tumor development in human oral mucosa. The animals were then used for biodistribution and pharmacokinetic studies of boronophenylalanine (BPA). Blood, tumor, precancerous pouch tissue surrounding tumor, normal pouch tissue, tongue, skin, cheek mucosa, palate mucosa, liver, and spleen, were sampled at 0–12 h after administration of 300 mg BPA/kg. The data reveal selective uptake of BPA by tumor tissue and, to a lesser degree, by precancerous tissue. Mean tumor boron concentration was 36.9 ± 17.5 ppm at 3.5 h and the mean boron ratios were 2.4:1 for tumor:normal pouch tissue and 3.2:1 for tumor:blood. Higher doses of BPA (600 and 1200 mg BPA/kg) increased tumor uptake. Potentially therapeutic absolute boron concentrations, and tumor:normal tissue and tumor:blood ratios can be achieved in the hamster oral cancer model using BPA as the delivery agent.

INTRODUCTION

BNCT is a binary treatment modality that involves the selective accumulation of \(^{10}\)B carriers in tumors followed by irradiation with a thermal or epithermal neutron beam. The high linear energy transfer \(\alpha\) particles and recoiling \(^{7}\)Li nuclei emitted during the capture of a thermal neutron by a \(^{10}\)B nucleus \((1, 2)\) have a range of approximately 5–9 \(\mu m\) in tissue and are known to have a high relative biological effectiveness \((3)\). Thus, BNCT would potentially target neoplastic tissue selectively \((4, 5)\). The basic requirements for a therapeutic advantage for BNCT are a high degree of selectivity for the accumulation of \(^{10}\)B in tumor relative to the surrounding normal tissues and a sufficiently high absolute concentration of \(^{10}\)B in tumor tissue \((6)\). As during any radiation therapy, the therapeutic tumor dose is limited by the tolerance of the surrounding normal tissue within the treatment volume \((7)\).

Clinical trials of BNCT for the treatment of glioblastoma multiforme and melanoma using BPA or BSH as the boron compounds are under way \((5, 8–11)\). Trials for brain tumors are in the dose-escalation phase in Europe \((Petten, Finland and, as of 2001, the Czech Republic and Sweden)\). In the United States, the initial Phase I clinical trials at Brookhaven National Laboratory and at Massachusetts Institute of Technology have been completed. The BNCT program at Brookhaven National Laboratory has ended, whereas there are plans to continue BNCT clinical trials at Massachusetts Institute of Technology. In Japan, intraoperative BNCT of brain tumours is performed using thermal beams and, more recently, the mixed thermal-epithermal beam of the JRR4 Reactor. Contributory experimental studies have been carried out using a variety of animal tumor models based on the implantation of tumor cells \((5, 12–18)\).

The current status of experimental and clinical BNCT studies warrants research on experimental models to devise strategies to improve the therapeutic advantage of this therapeutic modality, explore new applications, understand the biology and radiobiology of BNCT and analyze the behavior of clinically relevant normal tissues. Within this context we herein propose and validate the use of the hamster cheek pouch carcinogenesis model for BNCT studies. Hamster cheek pouch is the most widely accepted model of oral cancer \((19–22)\). Carcinogenesis protocols induce premalignant changes and SCCs that closely resemble human lesions \((23–26)\). The hamster cheek pouch anatomically resembles a pocket that is easily accessible to local tumor induction and can be readily everted for local treatment such as irradiation and macroscopic follow-up. A unique feature of this model lies in the possibility of undertaking macroscopic follow-up of the evolution of tumor, precancerous tissue, and normal tissue at different times after treatment by simply evert the cheek pouch and recording the chosen end points. Macroscopic end points could potentially be correlated with histopathological studies of biopsy samples. Vascular studies in particular can be performed elegantly on normal and neoplastic cheek pouch tissue using a variety of techniques \((27)\). Undoubtedly, the knowledge of the vascular changes involved in BNCT is critical to the effective application of this technique \((28–30)\).

Within the context of the search for new applications of BNCT, head and neck cancer may potentially benefit from the therapeutic advantage afforded by this modality. SCC of the head and neck is the sixth most prevalent cancer. Patients are generally treated with surgery combined with radiation therapy and chemotheraphy. Radical removal of a tumor and surrounding tissues results in large tissue defect \((31–34)\). Thus, tissue and organ preservation would be an important aim of alternative therapeutic strategies. The 5-year survival rate for head and neck SCC has improved only marginally over the past 20 years, remaining at 52% \((31)\). These data suggest the need for new therapeutic strategies \((35, 36)\).

Here we must stress that the approach of the present study is contributory solely from the viewpoint of basic research but may pave the way for future studies that could address the rationale for clinical application.

In terms of the study of the biology of BNCT the hamster cheek pouch model poses an advantage in that tumors are induced by a process that mimics the spontaneous process of malignant transfor-
MATERIALS AND METHODS

Boron Biodistribution and Pharmacokinetic Studies of BPA. Coderre et al. (6) in human glioblastoma multiforme. Furthermore, this model allows us to explore, in selected pouch tumor samples containing variable amounts of viable tumor tissue and small areas of necrosis, the possibility of addressing the issue of precancerous change involved in the process of field cancerization (39–43). The statistical significance of the differences between boron content of the different tissues at a given after administration time was assessed by a one-way ANOVA. An overall level of significance was taken to be α = 0.05. If the residuals were not normally distributed then logarithmic transformations of the data were performed to normalize the data. Histology and Tumor Viability. In keeping with the concept proposed by Coderre et al. (6) that BPA uptake depends on tumor cell viability, we examined the histology of representative tumors with different proportions of viable tumor tissue and analyzed the correlation with boron concentration. For this purpose each fresh tumor specimen was described macroscopically and divided into two contiguous samples of similar macroscopic appearance. 3.5 h after the administration of 300 mg BPA/kg b.w. One

Fig. 1. Hamster check pouch treated with DMBA for 16 weeks. The pouch has been everted to exhibit an exophytic tumor mass surrounded by precancerous tissue.

HAMSTER CHEEK POUCH AS AN ORAL CANCER MODEL FOR BNCT

Tumor Induction. The right cheek pouch of noninbred young (6 weeks old) Syrian hamsters was submitted to topical application of 0.5% DMBA in mineral oil three times a week for 14 weeks in keeping with a standard hamster cheek pouch carcinogenesis protocol (20). The protocol ensures humane practices. The treated pouch was periodically everted under light anesthesia to monitor tumor development. Once the exophytic tumors (Fig. 1) had developed and reached a diameter of approximately 3–5 mm, the animals were used for biodistribution and pharmacokinetic studies of BPA.

BPA Biodistribution Studies. BPA (l-enantiomer, >98% 10B-enriched; Boron Biologicals, Inc., Raleigh, NC) containing 4.9% B by weight was used as the boron delivery agent. Injection solutions of the BPA-fructose complex were prepared in keeping with procedures published previously (45, 46). A 0.14 M solution was prepared for i.p. administration and a 0.24 M solution was prepared for i.v. administration. In this way, convenient injection volumes were available for each administration route. Briefly, BPA was converted to a more soluble fructose complex by mixing BPA and fructose in water at a 1:1 molar ratio. The pH was adjusted to 9.5–10.0 with NaOH, and the mixture was stirred until all of the solids dissolved, and the pH was then readjusted to 7.4 with HCl. The concentration was then adjusted with USP water for injection to the corresponding molarity. The solution was passed through a 0.22-μm pore sterilization filter (Nalge Company, Rochester, NY). BPA was administered i.p. at a dose of 300, 600, or 1200 mg/kg b.w. Blood and tissue samples were removed at different post-administration times: 0, 0.5 h, 1 h, 1.5 h, 2.5 h, 3.5 h, 6 h, and 12 h for the dose of 300 mg BPA/kg b.w. On the basis of these data, the optimum time point in terms of absolute and relative tumor boron values was shown to be 3.5 h. The time point of 3.5 h was selected to assess boron delivery by the higher doses of BPA. Blood samples were taken from the jugular vein with a heparinized syringe. The following tissues were sampled: tumor, precancerous pouch tissue surrounding tumor (pouch treated with DMBMA), normal pouch tissue (nontreated pouch), tongue, cheek skin, cheek mucosa, palate mucosa, liver, and spleen. Noninjected animals were used as control. All of the samples were weighed immediately. Tissue samples were stored at −70°C, and blood samples were stored at 4°C until use.

A pharmacokinetic analysis was performed on the blood boron concentrations and time data after i.v. administration of 300 mg BPA/kg b.w. using a nonlinear regression program (WIN-NONLIN, professional 3.1). For this purpose we performed, in keeping with a technique developed previously by our laboratory (47), a bolus injection of the BPA solution in the surgically exposed jugular vein of animals anesthetized with an i.p. injection of ketamine (140 mg/kg) and xylazine (21 mg/kg) followed by skin suture. Survival from the surgical procedure was 100%.

Boron Analysis. Boron analysis was performed by ICP-AES. Tissue samples (≤50 mg) were digested at room temperature overnight (or at 60°C for 1 h) in 0.15 ml of a 1:1 mixture of concentrated sulfuric and nitric acids at a concentration of 50 mg tissue/ml acid solution; addition of 0.5 ml of a 10% solution of the detergent Triton X-100 and dilution to 1 ml with water resulted in a clear solution for ICP-AES analysis (6). Blood samples (200–300 μl) were prepared by adding 2.5% Triton X-100 at a final concentration of 0.1% in water (final volume 5 ml). Standard solutions of boric acid were used to prepare a calibration line during each day of operation. BPA biokinetics curves were obtained for blood and each of the tissues. The number of samples for each point ranged from 5 to 13. The statistical significance of the differences between boron content of the different tissues at a given after administration time was assessed by a one-way ANOVA. An overall level of significance was taken to be α = 0.05. If the residuals were not normally distributed then logarithmic transformations of the data were performed to normalize the data.

Fig. 2. Hamster cheek pouch treated with DMBA for 26 weeks. The pouch has been everted to exhibit an exophytic tumor mass surrounded by precancerous tissue.

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Histology was not attempted.

We dissected six areas of tumor that appeared to be characteristically necrotic on visual inspection and processed the tissues for boron analysis. For this purpose, each section occupied by viable tissue and necrotic tissue. The full extent of the histological section. We then evaluated the percentage area of tissue (tumor parenchyma and stroma), and necrotic tissue outlined on the image analyzer stained sections were analyzed by light microscopy. Areas of viable tumor fixed in buffered formalin, dehydrated, and embedded in paraffin. H&E-sample was analyzed for average boron content by ICP-AES. The other was /H11006 -11 samples; Control (noninjected) 0.7 /H9262 b

In the case of tumors, the range of values has been added in brackets. Results are expressed as mean SD. The number of samples per condition is indicated in brackets.

RESULTS

Mean boron concentration in blood for i.p. and i.v. bolus administration did not exhibit statistically significant differences over the 1–12 h post-administration time range (Table 1, Fig. 2) that would be relevant in terms of improving blood loading and clearance, except for a slight advantage for i.v. administration in terms of clearance at 6 h. The pharmacokinetic analysis showed that between 0 h and 12 h after administration of BPA, blood boron concentrations exhibited a decay that corresponds to the distribution (α) and elimination (β) phases in keeping with a model of two compartments. Calculation of the pharmacokinetic parameters afforded the following results: t1/2α = 0.21 ± 0.12 h; t1/2β = 3.30 ± 2.20 h; central compartment volume of distribution Vc = 502.8 ± 37.1 ml/kg; and apparent total body clearance Cl = 365.4 ± 145.5 ml/h.

For the dose of 300 mg BPA/kg b.w. administered i.p. (Table 1; Fig. 3), boron concentration in tumor was significantly higher than in normal pouch tissue (nontreated pouch) for 0.5 h (P = 0.021), 1 h

\[
\text{Time Post-Administration (h)} \quad \text{Blood (i.v.)} \quad \text{Blood (i.p.)} \quad \text{Tumor (i.p.)} \quad \text{Normal pouch tissue (i.p.)} \quad \text{Tissue around tumor (i.p.)} \quad \text{Cheek mucosa (i.p.)} \quad \text{Tongue (i.p.)} \quad \text{Palate mucosa (i.p.)} \quad \text{Cheek skin (i.p.)} \quad \text{Spleen (i.p.)} \quad \text{Liver (i.p.)}
\]

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<th>Time Post-Administration (h)</th>
<th>Blood (i.v.)</th>
<th>Blood (i.p.)</th>
<th>Tumor (i.p.)</th>
<th>Normal pouch tissue (i.p.)</th>
<th>Tissue around tumor (i.p.)</th>
<th>Cheek mucosa (i.p.)</th>
<th>Tongue (i.p.)</th>
<th>Palate mucosa (i.p.)</th>
<th>Cheek skin (i.p.)</th>
<th>Spleen (i.p.)</th>
<th>Liver (i.p.)</th>
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<td>Control (noninjected)</td>
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<td>11.7 ± 4.9</td>
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<td>12</td>
<td>1.6 ± 0.2</td>
<td>3.2 ± 2.1</td>
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<td>1.5 ± 1.3</td>
<td>1.1 ± 0.7</td>
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</table>

\[ a \text{ Results are expressed as mean ± S.D. The number of samples per condition is indicated in brackets.} \]

\[ b \text{ In the case of tumors, the range of values has been added in brackets.} \]

Fig. 2. Boron concentration (μg B/g) in blood versus time after either i.p. or i.v. injections of BPA at a dose of 300 mg/kg b.w. Each point represents 3–11 samples; bars, ± SD.

Fig. 3. Boron concentration (μg B/g) in blood, normal pouch tissue, tumor, and precancerous pouch tissue surrounding tumor versus time after i.p. administration of BPA at a dose of 300 mg/kg b.w. Each point represents 5–13 samples; bars, ± SD.
Table 2: Boron concentration ratios at different times after i.p. administration of 300 mg BPA/kg b.w.

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<th>1 h</th>
<th>1.5 h</th>
<th>2.5 h</th>
<th>3.5 h</th>
<th>6 h</th>
<th>12 h</th>
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<tr>
<td>Tumor/normal pouch</td>
<td>3.1</td>
<td>1.8</td>
<td>2.0</td>
<td>2.3</td>
<td>2.4</td>
<td>1.4</td>
<td>1.6</td>
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<tr>
<td>Tumor/blood</td>
<td>2.3</td>
<td>1.5</td>
<td>2.8</td>
<td>2.9</td>
<td>3.2</td>
<td>1.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Fig. 4. Boron concentration (µg B/g) in blood, cheek mucosa, palate mucosa, cheek skin, tongue, and normal pouch tissue versus time after i.p. administration of BPA at a dose of 300 mg/kg b.w. Each point represents 5–13 samples; bars, ± SD.

(p = 0.004), 2.5 h (p = 0.001), and 3.5 h (p = 0.000) after administration of BPA. At 1.5 h this difference did not reach statistical significance, a finding that could be attributed to the spread in values for tumor tissue. Boron concentration in tumor was higher than in blood at times of 0.5 h to 6 h. This difference reached statistical significance for 0.5 h (p = 0.043), 1 h (p = 0.027), 2.5 h (p = 0.018), 3.5 h (p = 0.000), and 6 h (p = 0.008).

The average tumor: normal pouch and tumor: blood ratios showed selective accumulation of BPA in tumor for all of the time points evaluated. The tumor: blood ratio improved with time after administration as blood clearance progressed. Table 2 shows the mean tumor: normal pouch and tumor: blood boron ratios for each time point. At 3.5 h the absolute boron value was 36.9 ± 17.5 ppm, and mean tumor: normal pouch tissue and tumor: blood ratios were 2.4:1 and 3:2:1 respectively. This would be the time point with the best therapeutic potential in terms of tumor absolute and relative boron concentration values, and normal tissue and blood boron content.

Precancerous tissue surrounding tumor (pouch treated with DMBA) showed a tendency, albeit not statistically significant, to concentrate BPA more than normal pouch tissue (Table 1; Fig. 3). Uptake of BPA in cheek mucosa, palate mucosa, cheek skin, and tongue did not differ significantly and exhibited the same tendency as in normal pouch tissue. Boron concentration rose in skin, intraoral tissues, and normal pouch tissue with time post-administration up to 3.5 h and began falling thereafter. In some cases, normal tissue boron values rose above concurrent blood values. This difference reached statistical significance for cheek mucosa at 2.5 h (p = 0.032; Table 1; Fig. 4). Liver and spleen boron concentration values were similar to those of normal pouch tissue and blood (Table 1). BPA accumulation in liver and spleen would, thus, not be a particular concern in this model.

The higher doses of 600 and 1200 mg BPA/kg b.w. administered i.p. delivered more boron to tumor tissue (Table 3). The normal tissue boron values for the dose of 600 mg BPA/kg b.w. were, overall, slightly higher than for the dose of 300 mg/kg b.w. The dose of 1200 mg delivered higher amounts of boron to normal tissues. Blood boron values for the two higher doses doubled the value for the 300 mg BPA/kg b.w. dose (Table 3; Fig. 5). The higher BPA doses may be therapeutically beneficial. Even if the tumor: blood boron concentration ratio remains fixed, the therapeutic ratio improves as more boron compound is administered, and higher absolute boron concentrations are produced in both tumor and normal tissues (5).

Boron concentration in tumor was markedly heterogeneous and ranged, on average, from 8.1 ppm in tumors composed of predominantly necrotic tissue to 32.0 ppm in tumors composed of mainly viable tissue in the set of tumor samples submitted to histological assessment and boron analysis (Fig. 6). Quantification of the proportion of viable tissue in 14 tumor sections showed a statistically significant (Student’s t test, P ≤ 0.01) difference in BPA uptake between moderately necrotic (18.5 ± 2.0 ppm) and mainly viable tumor tissue (32.0 ± 13.5 ppm). The only sample of mainly necrotic tissue large enough to perform a correlation between histology and ICP evaluation showed a markedly low boron uptake (8.1 ppm). The macroscopic description of the tumor closely agreed with the actual

![Figure 4](image4.png)

![Figure 5](image5.png)

Table 3: Boron concentration (ppm) in blood and tissue samples for different doses of BPA at 3.5 h after i.p. administration

<table>
<thead>
<tr>
<th>Dose of BPA</th>
<th>Blood</th>
<th>Tumor</th>
<th>Normal pouch tissue</th>
<th>Tissue around tumor</th>
<th>Cheek mucosa</th>
<th>Tongue</th>
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<td>300</td>
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<tr>
<td>600</td>
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a Results are expressed as mean ± S.D. The number of samples per condition is indicated in brackets.

b In the case of tumors, the range of values has been added in brackets.

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DISCUSSION

The present study proposes and validates the hamster cheek pouch carcinogenesis model for BNCT studies. It is possible to selectively deliver potentially therapeutic amounts of boron to hamster cheek pouch tumor by i.p. administration of BPA and achieve ratios between tumor and normal tissue and tumor and blood that would be compatible with treatment. The biodistribution data obtained in the present study indicates potentially therapeutic absolute and relative boron concentration values over the 1.5–3.5 h observation period. These findings fulfill the basic prerequisite of successful BNCT and, thus, render the model eligible for BNCT studies. Furthermore, alternative administration strategies in this model may provide an advantage. This issue may warrant future studies. I.v. bolus injection of BPA would not significantly improve blood loading and clearance as compared with i.p. administration in this model. Sequential injections may improve blood loading and tumor uptake. Two sequential intragastric doses have been reported to be additive in regard to loading tumors with boron in rat intracerebral gliosarcoma despite the fact that they do not increase the tumor:normal tissue and tumor: blood \(^{10}\)B concentration ratios (13, 14). Furthermore, it has been reported that longer i.v. infusion times are more effective in delivering boron selectively to tumor tissue in other models (48). Conceivably, i.v. infusion, as opposed to i.v. bolus administration, could improve selective uptake in hamster cheek pouch tumors.

BPA-mediated boron uptake in hamster cheek pouch tumors and tumor:normal tissue and tumor: blood ratios fall, overall, within the ranges reported in the literature for experimental animals and patients. Previous studies have reported that the average concentration of boron in normal brain is similar to that in the blood and that the average concentration of boron in glioma and melanoma is two to four times higher than in blood and brain (5, 6). The data reported herein for hamster tumor, normal tissue, and blood are in keeping with these values. The tissue: blood boron ratios for hamster skin and oral tissues closely resemble the scalp: blood (49) and skin: blood (44) ratios reported for BPA in patients. The blood and normal tissue boron values presented for the hamster oral cancer model fall within the range of blood and brain values reported for BPA in dogs (50). The biochemical rationale for the preferential uptake of BPA in tumor would be related to an elevated rate of amino acid transport at the tumor cell membrane (5). Within this context, the metabolic status of the cells may condition uptake and lead to a large spread in values. Heterogeneous uptake of BPA (5) holds true for hamster cheek pouch tumors and is an issue of concern in BPA-mediated BNCT.

The boron uptake data for precancerous tissue surrounding the tumor would evidence a tendency of premalignant tissue to incorporate more boron compound than normal tissue. This finding would provide a rationale for treatment of “field cancerized” tissue surrounding tumor (38) to reduce the risk of development of additional tumors in the area. However, because this particular tissue may incorporate more boron than normal tissue, its dose-limiting nature in terms of acute effects must be explored. The fact that the hamster oral cancer model can be used to study precancerous tissue is an advantage over transplanted tumor models.

This model allows us to study skin and intraoral tissues in addition to actual pouch tissue. Furthermore, the fact that boron concentration is similar in all of these tissues confers an additional advantage on the normal pouch tissue as a model of normal intraoral tissues and skin in terms of boron uptake. Given that boron concentration may rise above...
blood values in some cases in these normal tissues, it would be of utmost importance to evaluate, or at least estimate, actual uptake of boron by oral mucosa rather than extrapolate from blood values in patients if the present findings on normal oral tissues hold true for human subjects. BNCT treatment protocols in Europe are in the dose escalation phase. Whereas these protocols do not yet entail acute radiation damage to the oral mucosa, it could become a potential dose-limiting tissue in future treatment protocols involving higher doses, larger target irradiation field sizes, or multiple fields (7). The study of clinically relevant, potentially dose-limiting normal tissues in an adequate animal model such as the model described herein may contribute to elucidating previously unpredictable side-effects.

Our observations regarding high boron uptake in mainly viable tumor tissue and low boron uptake in mainly necrotic tumor tissue are in keeping with other studies (6). Moreover, the data of Patel and Sedgwick, (51) show that BPA accumulates preferentially in viable cells with uptake capacity. Conversely, BSH that has no known uptake mechanisms and is thought to penetrate a tumor by diffusion and leakage through tumor blood vessels would accumulate more in necrotic areas. The data reported herein would allow us to assume that BPA uptake values for 100% viable tumor tissue will be closer to the maximum values than to the means. In this sense, actual tumor:normal tissue and tumor: blood ratios for viable cells may be higher than the mean ratios. Thus, we could speculate that the therapeutic advantage of BCNT would increase with tumor viability in this model. Heterogeneous uptake by heterogeneous tumor tissues (52, 53) precludes accurate treatment planning. The agreement between prescribed and obtained 10B concentration during irradiation. A large spread in boron concentrations creates additional problems in the analysis of biological responses (54). Once again, the possibility of addressing these issues experimentally in a model such as the hamster cheek pouch could contribute to improving the therapeutic advantage of BNCT. In particular, studies currently underway will examine, in more detail, the correlation between compound uptake and tumor histology. Tumor viability is central to uptake as shown by Coderre (7) has reported a compound biological effectiveness factor of 4.9 for BPA in normal rat tongue. The compound biological effectiveness factor for hamster cheek pouch remains to be determined. This model would be particularly useful to evaluate the response of dose-limiting oral tissues and assess the sparing effects of split-dose or fractionated BNCT irradiation schedules. The hamster cheek pouch model would allow for definitive scoring of tumor, precancerous, and normal tissue reaction to BNCT. This model also allows for the study of the role of vascularization in the BNCT-induced effect. The effect of local x-irradiation on vascularization has been studied by other authors in this model (55).

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