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"Sequential" Boron Neutron Capture Therapy (BNCT): A Novel Approach to BNCT for the Treatment of Oral Cancer in the Hamster Cheek Pouch Model

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In the present study the therapeutic effect and potential toxicity of the novel "Sequential" boron neutron capture therapy (Seq-BNCT) for the treatment of oral cancer was evaluated in the hamster cheek pouch model at the RA-3 Nuclear Reactor. Two groups of animals were treated with "Sequential" BNCT, i.e., BNCT mediated by boronophenylalanine (BPA) followed by BNCT mediated by sodium decahydrodecaborate (GB-10) either 24 h (Seq-24h-BNCT) or 48 h (Seq-48h-BNCT) later. In an additional group of animals, BPA and GB-10 were administered concomitantly [(BPA + GB-10)-BNCT]. The single-application BNCT was to the same total physical tumor dose as the "Sequential" BNCT treatments. At 28 days post-treatment, Seq-24h-BNCT and Seq-48h-BNCT induced, respectively, overall tumor responses of 95 ± 2% and 91 ± 3%, with no statistically significant differences between protocols. Overall response for the single treatment with (BPA + GB-10)-BNCT was 75 ± 5%, significantly lower than for Seq-BNCT. Both Seq-BNCT protocols and (BPA + GB-10)-BNCT induced reversible mucositis in the dose-limiting precancerous tissue around treated tumors, reaching Grade 3/4 mucositis in 47 ± 12% and 60 ± 22% of the animals, respectively. No normal tissue toxicity was associated with tumor response for any of the protocols. "Sequential" BNCT enhanced tumor response without an increase in mucositis in dose-limiting precancerous tissue.

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INTRODUCTION

Boron neutron capture therapy (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 minor abundance stable isotope of boron, ^{10}B, interacts with low-energy (thermal) neutrons to produce high-linear energy transfer (LET) α particles and ^{7}Li ions with high relative biological effectiveness (RBE). Their short range (<10 μm) would limit the damage to the cells containing ^{10}B (*1, 2*). Thus BNCT would target tumor tissue selectively, sparing normal tissue.

Clinical trials of BNCT for the treatment of glioblastoma multiforme, melanoma and, more recently, head and neck tumors and liver metastases using boronophenylalanine (BPA) or sodium mercaptoundecahydrododecaborane (BSH) as the ^{10}B carriers have been performed or are under way in Argentina, Japan, the U.S. and Europe (*e.g. 3–8*). To date, the clinical results have shown a possible, albeit inconclusive, therapeutic advantage for this technique. Related translational studies have been carried out employing a variety of transplanted tumor models (*e.g. 5*).

The use of the hamster cheek pouch model of oral cancer was previously proposed and subsequently validated by our group to explore new applications of BNCT, study its radiobiology and improve its therapeutic efficacy (*9*). The clinical relevance of the search for new therapeutic strategies for head and neck squamous cell carcinoma (SCC) lies in the relatively poor 5-year survival rate for advanced head and neck SCC and the large tissue defect caused by radical surgery (*10*). The hamster cheek pouch model is widely accepted as a model of oral cancer (*11*). Carcinogenesis protocols induce premalignant and malignant changes that closely resemble spontaneous human oral mucosa lesions (*12*).

The hamster cheek pouch model of oral cancer has a unique advantage in that tumors are induced by periodic topical application of the carcinogen dimethyl-1,2-benzanthracene (DMBA), a process that mimics the spontaneous process of malignant transformation. In contrast, rodent tumor models usually employed in small-animal BNCT studies are based on the growth of implanted cancer cells in healthy tissue (*e.g. 5*). In the hamster cheek pouch, the carcinogenesis protocols lead to the development of what has been called ''precancerous tissue'' (*e.g. 9*) or ''tissue with potentially malignant disorders (PMD)'' (*13*), from which tumors arise. Thus this mode of tumor induction provides tumor surrounded by precancerous tissue. Studying precancerous tissue in addition to tumor and normal tissue is clinically relevant in terms of potentially dose-limiting tissue. The fact that the carcinogenesis process in this model mimics the process that takes place in human oral cancer is an advantage. However, it imposes restrictions on the end points that can be used to evaluate response to BNCT. The development of tumors from the precancerous tissue precludes use of the model to assess tumor recurrence and long-term tumor control. Furthermore, liver injuries caused by multiple DMBA applications can cause morbidity (seen as weight loss and ascitis) and preclude long-term follow-up (*9, 11, 13*). During the 28-day period used in the present study, the toxicity was not a problem. However, any potential influence of liver alterations would be accounted for by comparison of experimental groups treated with the same carcinogenesis protocol.

Previous studies have demonstrated that BPA delivers therapeutically useful amounts of ^{10}B to SCC in the hamster cheek pouch (*9*) and showed the success of BNCT mediated by BPA to treat hamster cheek pouch tumors with no normal tissue toxicity (*14*). BPA-BNCT has been used to treat spontaneous SCC in cats (*15, 16*).

Different strategies have been proposed to optimize the therapeutic advantage of BNCT and circumvent potential problems. Because targeting of all tumor populations within a heterogeneous tumor is critical to the success of BNCT (or any oncological therapy), it has been postulated that the combined administration of different boron compounds with different properties and complementary uptake mechanisms may enhance the therapeutic efficacy of BNCT (*17–19*). The therapeutic efficacy of BNCT mediated by sodium decahydrodecaborate ($Na_2{}^{10}B_{10}H_{10}$, known as GB-10) and by the combination of GB-10 and BPA was explored (*22*). GB-10 forms the anion $[B_{10}H_{10}]^{2-}$ in aqueous solution and is diffusive in nature. GB-10 was initially proposed for the treatment of brain tumors. Given that GB-10 does not cross the intact blood-brain barrier (BBB), selective uptake by the tumor would have to rely on an intact BBB in normal tissue and a disrupted BBB in the tumor (*20, 21*). In the hamster cheek pouch model, GB-10 was able to deliver therapeutically useful amounts of boron to the tumors, albeit not selectively (*22*). Unlike BPA (*9*), GB-10 was deposited homogeneously in different tumor areas, an asset when treating heterogeneous tumors (*19*). In contrast to the traditional BNCT paradigm that posits that selective damage to tumor is based on selective tumor uptake of the boron compound, previous studies demonstrated that BNCT mediated by GB-10 acted selectively on tumors by damaging tumor blood vessels while sparing blood vessels in precancerous and normal tissue (*18, 23*). The structure and function of angiogenic tumor blood vessels are altered (*24, 25*), rendering them more sensitive to BNCT than the precancerous and normal tissue blood vessels, and thereby providing a selective mechanism of action for a chemically non-selective boron compound (*18, 23, 26*). In contrast to GB-10, BPA is incorporated selectively by tumors, albeit heterogeneously (*9, 19*). BPA-BNCT would act directly on tumor cells, whereas GB-10-BNCT would act indirectly on tumor cells, secondary to blood vessel damage (*18*). BNCT mediated by BPA and GB-10 administered concomitantly would combine cellular targeting and vascular targeting to achieve improved tumor response with no normal tissue toxicity and reversible albeit potentially dose-limiting mucositis in precancerous tissue (*18, 26*). The vascular targeting effect of GB-10-BNCT would differ from the effect of anti-angiogenic agents that must be administered repeatedly (*25*). GB-10-BNCT appears to induce tumor cell death largely by endothelial cell damage and ensuing tissue ischemia similar to the mechanism described for photodynamic therapy (*18*). Previous BNCT studies in the hamster cheek pouch model in the RA-3 Nuclear Reactor thermal column showed marked therapeutic efficacy but left room for improvement (*26*). Dose escalation is limited by mucositis in the dose-limiting precancerous tissue. Despite the differences between clinical head and neck cancer studies and experimental studies in the hamster cheek pouch model, experimental findings could contribute to the understanding of the problems likely to be encountered in BNCT studies with head and neck cancer (*e.g. 8*). Confluent oral mucositis is a frequent dose-limiting side effect during conventional radiotherapy for advanced head and neck tumors (*2, 27*), affecting approximately 80% of patients (*28*). No effective way to prevent or treat mucositis is currently available (*27, 29*). Oral mucositis is a dose-limiting toxicity in BNCT of head and neck tumors (*2, 8*). BNCT protocols that minimize mucositis are more likely to deliver therapeutic doses to a tumor without exceeding tissue tolerance.

The aim of the present study was to explore the tumor response and potential toxicity of a novel approach to BNCT termed ''Sequential'' BNCT based on the sequential application of BPA-BNCT followed by GB-10-BNCT with an interval of 24 or 48 h between the two treatments.

The working hypothesis was that this strategy would improve therapeutic effect without causing unacceptable toxicity in normal or dose-limiting precancerous tissue.

MATERIALS AND METHODS

Tumor Induction

The right cheek pouches of non-inbred 6-week-old Syrian hamsters received topical application of 0.5% dimethyl-1,2-benzanthracene (DMBA) in mineral oil twice a week for approximately 12 weeks according to a modification of a standard hamster cheek pouch carcinogenesis protocol (*30*). Institutional and national guidelines were followed during the performance of the studies. The studies were reviewed and approved by the committee of the National Atomic Energy Commission (Argentina) that oversees the ethics of research involving animals. The treated pouch was periodically everted under light ketamine-xylazine anesthesia and examined to monitor tumor development. Once the exophytic tumors had developed and reached a diameter of approximately ≥1 mm, the animals were used for BNCT studies.

In Vivo BNCT

Irradiations were performed at a novel neutron source constructed for use in BNCT biomedical applications by the National Atomic Energy Commission of Argentina at the RA-3 research and production nuclear reactor facility located in Buenos Aires (*31*). A tunnel penetrating the graphite structure of the thermal column enables the insertion of samples into a near-isotropic neutron field while the reactor is in normal operation. The neutron field is very well thermalized, making the radiation dose component from hydrogen recoil (i.e. fast neutron dose) in tissue negligible. A shield was constructed to protect the body of the animal from the thermal neutron flux while exposing the everted cheek pouch bearing tumors. The enclosure was fabricated from plates composed of a 6-mm layer of lithium carbonate enriched to 95% in lithium-6, sealed within sheets of Lucite. The hamster pouch was everted out of the enclosure onto a protruding shelf. The temperature at the irradiation site within the tunnel was approximately 30°C. No action was taken to keep the tumors moist during the short irradiations (3.13 to 6.42 min). Physical dosimetry data for the irradiation system have been reported previously (*26*). Briefly, the thermal neutron flux is about 8.2×10^9 n cm^{-2} s^{-1} in the outermost position on the pouch shelf and 7×10^9 n cm^{-2} s^{-1} in the center position. These values are approximately 25% lower than the free flux at this location, largely due to local flux depression by the shield enclosure. The thermal neutron flux at all locations within the shield was at least a factor of 20 lower than the flux on the pouch shelf. The dose rate of γ rays in air at the irradiation location was 6.5 ± 0.5 Gy h^{-1}.

One group of hamsters ($n = 8$) bearing a total of 128 tumors was treated with BNCT mediated by BPA followed by BNCT mediated by GB-10 24 h later (Seq-24h-BNCT). A second group of hamsters ($n = 9$) bearing a total of 92 tumors was treated with the same BNCT applications, but 48 h apart (Seq-48h-BNCT). The total physical dose prescribed to tumor in both cases was 9.93 Gy. The actual absorbed physical dose may differ from the prescribed physical dose due to variations in boron content. For BPA-BNCT, BPA (0.14 *M*) was administered intraperitoneally (i.p.) at a dose of 15.5 mg ^{10}B/kg body weight. GB-10 (generously provided by Neutron Therapies LLC, San Diego, CA) was administered at a dose of 50 mg ^{10}B/kg body weight as a bolus injection in the surgically exposed jugular vein under i.p. ketamine (140 mg/kg body weight)–xylazine (21 mg/kg body weight) anesthesia. The animals were irradiated 3 h after administration of the corresponding boron compound under i.p. ketamine (140 mg/kg bw)–xylazine (21 mg/kg bw) anesthesia. A third group of hamsters ($n = 5$) bearing a total of 35 tumors was treated with sequential beam-only (BO) irradiations 24 h apart (Seq-24h-BO). Finally, a fourth group of hamsters ($n = 2$) bearing a total of 31 tumors was treated with sequential beam-only irradiations 48 h apart (Seq-48h-BO).

A fifth group of animals ($n = 5$) bearing a total of 76 tumors was treated with a single application of BNCT mediated by the combined administration of GB-10 and BPA. The total physical dose to tumor was matched with that delivered with the Seq-BNCT protocols (9.93 Gy) and was higher than the dose used in previous studies (*18*). For the (BPA + GB-10)-BNCT protocol, BPA (0.14 *M*) was administered i.p. at a dose of 15.5 mg ^{10}B/kg body weight and GB-10 was administered intravenously (i.v.) at a dose of 50 mg ^{10}B/kg body weight shortly thereafter. Previous biodistribution studies in this model demonstrated no significant differences in boron biodistribution when BPA was administered i.p. or i.v. (*9*). When GB-10 and BPA were administered concomitantly (*22*), GB-10 was administered i.v. to avoid volume overload in the peritoneal cavity. A pilot study indicated that i.v. combined administration of both compounds was badly tolerated by the animals. The animals were irradiated 3 h after the administration of the boron compounds. A sixth group of animals ($n = 3$) bearing a total of 35 tumors was treated with a single beam-only irradiation. All the beam-only irradiations were performed to evaluate the effect of the background dose (γ rays and ^{14}N thermal neutron capture-induced protons) of the BNCT treatments and were matched for irradiation time with the corresponding BNCT treatments.

The right normal pouches of animals that had not been treated with the carcinogenesis protocol were also treated with each protocol to evaluate potential normal tissue toxicity ($n = 5$/protocol).

Dosimetric calculations were based on previously reported boron biodistribution data for the administration protocols used (*9, 19, 22*). Briefly, at the established times after administration of the boron compounds, samples of blood, tumor, precancerous pouch tissue and clinically relevant normal tissues were taken for each animal. Tissue and blood samples were processed for boron analysis by Inductively Coupled Plasma–Atomic Emission Spectroscopy (ICP-OES Optima 3100 XL, UV, axial, Perkin Elmer). Tissue samples (30–50 mg) were digested for 1 h at 100°C in 0.25 ml of a 1:1 mixture of concentrated sulfuric and nitric acids. Once the digestion process was complete, 0.2 ml yttrium (0.5 ppm)-strontium (25 ppm) was added as an internal standard prior to the addition of 0.55 ml of a 5% Triton X-100 solution in water. The samples were then sonicated for 90 min. Blood samples (200–300 µl) were digested at 100°C in 1.25 ml of a 1:1 mixture of concentrated sulfuric and nitric acids. Once the digestion process was complete, 1 ml yttrium (0.5 ppm)-strontium (25 ppm) was added as an internal standard prior to the addition of 2.75 ml of a 5% Triton X-100 solution in water. Standard solutions of boric acid (enriched to 99.8% ^{10}B) were used to prepare a calibration curve each day. Boron measurements were performed using the boron 249.677-nm analytical line. Absolute boron concentrations in tumor, blood and clinically relevant normal tissues were measured for each protocol. These previous biodistribution studies also showed that the boron concentration in blood and tissues 24 h after the administration of BPA was negligible. Thus BPA was considered not to contribute to boron levels after administration of GB-10 in the "Sequential" protocols. Additional studies were performed to evaluate the potential influence of prior BPA-BNCT on GB-10 uptake in tumor and precancerous tissue 24 and 48 h later. Boron measurements were made by three methods: ICP-OES, inductively coupled plasma mass spectroscopy (ICP-MS), and neutron autoradiography (*6*). All three methods showed consistent results and did not reveal therapeutically relevant changes in GB-10 uptake in animals previously treated with BPA-BNCT or in the roughly 1:1 tumor/precancerous tissue boron ratio reported previously for GB-10 in unirradiated animals (*22*).

Follow-up

The tumor and precancerous tissue responses were assessed by visual inspection and tumor volume assay before treatment and at 2,

TABLE 1
Boron Concentration (mean ± SD) (ppm) for the Different Administration Protocols

	BPA[a]	GB-10[b]	GB-10[c] + BPA[d]
Tumor	33 ± 17	32 ± 21	43 ± 9
Precancerous tissue	20 ± 6	34 ± 17	50 ± 10
Normal pouch tissue	14 ± 5	22 ± 7	42 ± 7
Blood	12 ± 4	32 ± 6	41 ± 11

[a] 3 h after 15.5 mg ^{10}B/kg body weight bolus i.p.
[b] 3 h after 50 mg ^{10}B/kg body weight bolus i.v.
[c] 3 h after 50 mg ^{10}B/kg body weight bolus i.v.
[d] 3.5 h after 15.5 mg ^{10}B/kg body weight bolus i.p.

7, 14, 21 and 28 days post-treatment. Tumor volume was determined by external caliper measurement of the three largest orthogonal diameters (d) and calculated as $d_1 \times d_2 \times d_3$ (*14, 18*). A reduction from initial tumor volume was considered as partial response (PR) as defined previously (*14, 18*). A reduction to ≤50% of the initial tumor volume was called a partial response$_{0.5}$ ($PR_{0.5}$). Complete tumor response (CR) was defined as disappearance of the tumor on visual inspection and no evidence of tumor on histological analysis. Overall response (OR) was defined as PR + CR in keeping with previous studies (*14, 18*) and overall response$_{0.5}$ ($OR_{0.5}$) was defined as $PR_{0.5}$ + CR. The animals in the Seq-BNCT protocols were also examined prior to the second application. The normal left pouch (not treated with the carcinogen and shielded during the irradiation) was examined alongside the carcinogen-treated, radiation-exposed pouch. The right pouches of the animals not treated with the carcinogenesis protocol but treated with the same protocols as tumor-bearing hamsters were examined at the same times and then weekly for 6 months post-treatment. To evaluate toxicity, clinical signs and body weights for all the animals were monitored regularly. To evaluate tumor control and pouch tissue toxicity, tumor-bearing animals were examined 2, 7, 14, 21 and 28 days post-treatment. Normal animals (not treated with the carcinogenesis protocol) were monitored at the same times and weekly thereafter up to 6 months post-treatment. The severity of mucositis was evaluated semiquantitatively according to an oral mucositis scale based on macroscopic features, adapted for the carcinogen-treated hamster cheek pouch from the WHO classification for oral mucositis in human subjects (*32*) and the six-point grading system for normal hamster cheek pouches of Sonis *et al.*

TABLE 2
Physical Prescribed Doses (Gy) for the Different Experimental Protocols

	Gamma rays	Boron (tumor)	Boron (normal tissue)	Induced protons
BPA (15.5 mg ^{10}B/kg body weight, bolus i.p.)-BNCT (effective irradiation time: 3.13 min)				
Pouch	0.35 ± 0.04	0.10 ± 0.01	0.10 ± 0.01	0.28 ± 0.02
Body	0.35 ± 0.04		0.00 ± 0.00	0.01 ± 0.00
GB-10 (50 mg ^{10}B/kg body weight)-BNCT (effective irradiation time: 4.96 min)				
Pouch	0.55 ± 0.07	0.16 ± 0.01	0.16 ± 0.01	0.44 ± 0.04
Body	0.55 ± 0.07		0.01 ± 0.00	0.02 ± 0.00
[GB-10 (50 mg ^{10}B/kg body weight) + BPA (15.5 mg ^{10}B/kg body weight)]-BNCT (effective irradiation time: 6.42 min)				
Pouch	0.71 ± 0.08	0.20 ± 0.02	0.20 ± 0.02	0.57 ± 0.05
Body	0.71 ± 0.08		0.01 ± 0.00	0.02 ± 0.00

Note. Boron dose components are quoted as part per million boron.

TABLE 3
Total Prescribed Physical Doses (Gy)

	BPA-BNCT	GB-10-BNCT	(GB-10 + BPA)-BNCT
Pouch tumor	3.92 ± 1.75	6.00 ± 3.39	9.93 ± 1.91
Precancerous tissue	2.57 ± 0.60	6.37 ± 2.69	11.30 ± 2.14
Normal pouch tissue	2.05 ± 0.50	4.38 ± 1.14	9.76 ± 1.58
Body	0.41 ± 0.05	0.73 ± 0.14	1.03 ± 0.17

(*33*), i.e., Grade 0: healthy appearance, no erosion or vasodilation; Grade 1: erythema and/or edema and/or vasodilation, no evidence of mucosal erosion; Grade 2: severe erythema and/or edema, vasodilation and/or superficial erosion; Grade 3: severe erythema and/or edema, vasodilation and formation of ulcers <2 mm in diameter; Grade 4: severe erythema and/or edema, vasodilation and formation of ulcers >2 mm in diameter; Grade 5: virtually complete ulceration of the pouch mucosa. Grading was based on the most severe feature observed, avoiding areas close to persistent tumors and the pouch *cul de sac* that is histologically different from the rest of the pouch, overly radiosensitive and of limited clinical relevance.

At the last time evaluated (28 days post-treatment) the animals were killed humanely for histological analysis of persistent tumors, precancerous tissue surrounding treated tumors, and the contralateral (shielded) normal pouch tissue. Normal animals (not treated with the carcinogenesis protocol) treated with the different protocols were killed humanely at 28 days or 6 months post-treatment for analysis of tissue short-term and long-term toxicity to the normal pouch at the histological level.

Statistical analysis of differences in tumor response was performed using Fisher's exact test. Statistical significance was set at $P = 0.05$.

RESULTS

The boron concentration data relevant to this study are summarized in Table 1. Boron concentrations in tumors fell within a therapeutically useful range (*18*). Table 2 presents the prescribed physical doses from the different radiation components and the corresponding irradiation times for the different treatment protocols, and Table 3 shows the corresponding total physical prescribed doses. The doses for BPA-BNCT and GB-10-BNCT (which when added give the "Sequential" BNCT dose) are presented separately. The total beam-only tumor dose was 1.62 Gy for the "Sequential" protocols and 1.28 Gy for the single application protocol.

Tumor response data at 28 days post-treatment are presented in Table 4. Three arbitrary tumor sizes (small: <10 mm^3, medium: 10–100 mm^3, large: >100 mm^3) were used to categorize tumor sizes at the time of irradiation and evaluate variations in response with size (*4, 23*). The Seq-24h-BNCT and Seq-48h-BNCT protocols induced a similar overall tumor response, with no statistically significant difference between the "Sequential" BNCT protocols for any response end point. Tumor response after a single application of (BPA+GB-10)-BNCT was significantly lower than for Seq-24h-BNCT and Seq-48h-BNCT.

For both "Sequential" BNCT protocols, the incidence of complete response was significantly higher for the small tumors than for the medium and large tumors

TABLE 4
Tumor Response: BNCT Protocols

Tumors	*n*	Complete response (% ± SE)	Partial response (PR) as reduction from initial volume (% ± SE)	Partial response as reduction to 50% of initial volume ($PR_{0.5}$) (% ± SE)	No response (% ± SE)	Overall response (PR + CR) (% ± SE)
Sequential BNCT 24 h						
BPA (15.5 mg ^{10}B/kg body weight)-BNCT followed by GB-10 (50 mg ^{10}B/kg body weight)-BNCT 1 day later						
Total	128	76 ± 4	19 ± 3	18 ± 3	5 ± 2	**95 ± 2**
Large (>100 mm^3)	11	9 ± 9	82 ± 12	73 ± 13	9 ± 9	**91 ± 9**
Medium (10–100 mm^3)	27	70 ± 9	26 ± 8	26 ± 8	4 ± 4	**96 ± 4**
Small (<10 mm^3)	90	87 ± 4	9 ± 3	9 ± 3	4 ± 2	**96 ± 2**
Sequential BNCT 48 h						
BPA (15.5 mg ^{10}B/kg body weight)-BNCT followed by GB-10 (50 mg ^{10}B/kg body weight)-BNCT 2 days later						
Total	92	68 ± 5	23 ± 5	20 ± 4	9 ± 3	**91 ± 3**
Large (>100 mm^3)	8	25 ± 15	63 ± 17	63 ± 17	12 ± 11	**88 ± 11**
Medium (10–100 mm^3)	21	52 ± 11	43 ± 11	33 ± 10	5 ± 5	**95 ± 5**
Small (<10 mm^3)	63	79 ± 5	11 ± 4	10 ± 4	10 ± 4	**90 ± 4**
Single application BNCT						
[BPA (15.5 mg ^{10}B/kg body weight) + GB-10 (50 mg ^{10}B/kg body weight)]-BNCT						
Total	76	50 ± 6	25 ± 4	17 ± 4	25 ± 5	**75 ± 5**
Large (>100 mm^3)	7	43 ± 19	43 ± 19	43 ± 19	14 ± 13	**86 ± 13**
Medium (10–100 mm^3)	13	39 ± 14	38 ± 10	15 ± 10	23 ± 12	**77 ± 12**
Small (<10 mm^3)	56	53 ± 7	20 ± 5	14 ± 5	27 ± 6	**73 ± 6**

($P = 0.0001$ for Seq-24h-BNCT and $P = 0.0015$ for Seq-48h-BNCT). The effect of the initial size of the tumor was similar for both "Sequential" BNCT protocols. In the case of the single application of (BPA+GB-10)-BNCT, the difference in the complete response for small tumors and for medium and large tumors was not statistically significant.

The Sequential-BO protocols produced no complete tumor responses. The partial responses for the Seq-24h-BO and Seq-48h-BO protocols were similar. Overall tumor $response_{0.5}$ showed no statistically significant difference between the "Sequential" protocols. The single beam-only protocol induced a slightly higher (but not statistically significant) overall tumor response and overall tumor $response_{0.5}$ and no complete responses (Table 5).

A light microscopy analysis of the histology of the few tumors that were present 28 days after "Sequential" BNCT and of the tumors present 28 days after single application of (BPA+GB-10)-BNCT revealed consistent differences. It must be remembered that only persistent tumors that responded poorly to therapy were available for histological evaluation 28 days post-treatment. The tumors that persisted 28 days after Seq-BNCT were predominantly less infiltrating and more differentiated than the tumors that persisted 28 days after a single application of (BPA+GB-10)-BNCT. They exhibited less atypia, were richer in stroma, exhibited larger areas of fibrosis interspersed between areas of viable tumor cells, and were overall less aggressive (Fig. 1). A semi-quantitative, subjective estimation of the number of tumors that fitted the description of "more differentiated" was performed for each of the BNCT protocols and yielded the following ratios: 13/19 tumors for the Seq-24h-BNCT protocol, 10/12 tumors for the Seq-48h-BNCT protocol, 23/31 tumors for the Seq-BNCT protocols taken together, and 2/5 tumors for the single application BNCT protocol. Due to the small sample size for the single application BNCT protocol, the findings are described as only a trend.<figure-placer number= 1">

The Seq-BNCT protocols and (BPA + GB-10)-BNCT induced reversible mucositis in the dose-limiting precancerous tissue around treated tumors that peaked at 14 days post-treatment and had resolved by 21–28 days post-treatment (Fig. 2). The incidence of Grade 3/4 mucositis at 14 days post-treatment was 35 ± 12% for the Seq-BNCT protocols taken together (no statistically significant differences were observed between "Sequential" protocols) and 60 ± 22% for the (BPA+GB-10)-BNCT protocol (Fig. 2). Histological analysis of precancerous tissue 28 days post-treatment also indicated that mucositis had resolved. Overall mucositis (over the whole study period) in precancerous tissue around treated tumors reached Grade 3/4 in 47 ± 12% of the animals in the two Seq-BNCT protocols taken together (no statistically significant differences were observed between "Sequential" protocols) and in 60 ± 22% of the animals in the (BPA+GB-10)-BNCT protocol. The difference in mucositis in precancerous tissue in the Seq-BNCT and (BPA+GB-10)-BNCT protocols was not statistically significant. The Seq-BNCT protocols did not cause greater toxicity in precancerous tissue. None of the beam-only protocols induced more than Grade 2 mucositis in precancerous tissue.

TABLE 5
Tumor Response: Beam-Only Protocols

Tumors	*n*	Complete response (CR) (% ± SE)	Partial response (PR) as reduction from initial volume (% ± SE)	Partial response as reduction to ≤50% of initial volume ($PR_{0.5}$) (% ± SE)	No response (% ± SE)	Overall response (PR + CR) (% ± SE)
Sequential beam only 24 h						
Beam only followed by beam only 1 day later						
Total	34	0	3 ± 3	0	97 ± 3	**3 ± 3**
Large (>100 mm^3)	2	0	0	0	100 ± 21	**0**
Medium (10–100 mm^3)	7	0	14 ± 13	0	86 ± 13	**14 ± 13**
Small (<10 mm^3)	25	0	0	0	100 ± 6	**0**
Sequential beam only 48 h						
Beam only followed by beam only 2 days later						
Total	31	0	6 ± 4	6 ± 4	94 ± 4	**6 ± 4**
Large (>100 mm^3)	1	0	0	0	100 ± 30	**0**
Medium (10–100 mm^3)	4	0	25 ± 22	25 ± 22	75 ± 22	**25 ± 22**
Small (<10 mm^3)	26	0	4 ± 4	4 ± 4	96 ± 4	**4 ± 4**
Single application						
Beam only						
Total	35	0	14 ± 6	11 ± 5	86 ± 6	**14 ± 6**
Large (>100 mm^3)	1	0	100 ± 30	0	0	**100 ± 30**
Medium (10–100 mm^3)	10	0	30 ± 14	30 ± 14	70 ± 14	**30 ± 14**
Small (<10 mm^3)	24	0	4 ± 10	4 ± 10	96 ± 10	**4 ± 10**

No macroscopic mucosal effects were observed in normal pouches exposed to any of the protocols at 28 days or 6 months post-treatment (results not shown). The contralateral normal pouch tissue that was shielded during irradiation did not show any macroscopic or microscopic signs of toxicity at any of the times evaluated for any of the protocols. No changes were observed in the health status of the treated animals. Body weights oscillated slightly over the post-treatment period, with variations (gains or losses) that did not exceed on average 10% of the initial weight.

DISCUSSION

Having demonstrated the therapeutic efficacy of BPA-BNCT, GB-10-BNCT and (BPA + GB-10)-BNCT in hamster cheek pouch tumors with no normal tissue toxicity but potentially dose-limiting mucositis in precancerous

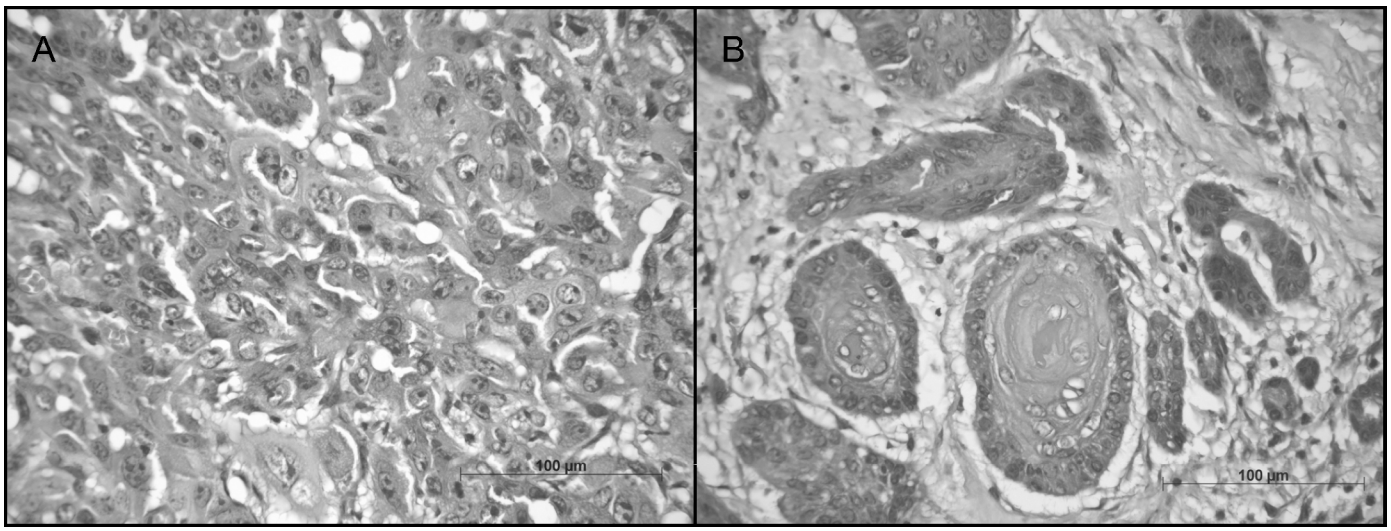

FIG. 1. Characteristic light microscopy images of carcinomas that persisted 28 days after treatment. Panel A: Single application of (BPA+GB-10)-BNCT. The tumor is very aggressive and undifferentiated and exhibits infiltrating cords, multiple features of atypia and scarce stroma. Panel B: "Sequential" BNCT (Seq-24h-BNCT). The tumor is differentiated and exhibits loosely packed cords with abundant stroma. Hematoxylin-eosin stain.

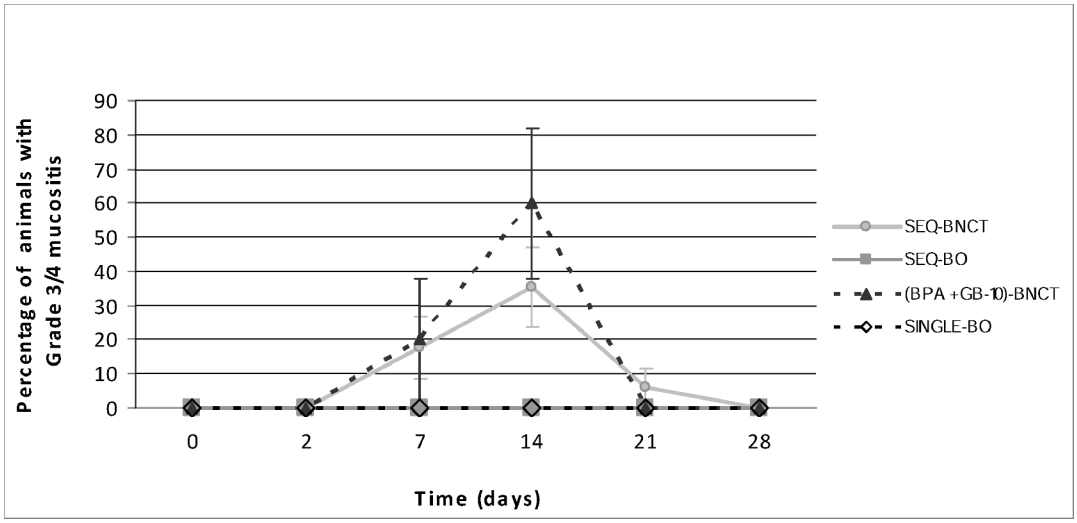

FIG. 2. Incidence of animals with Grade 3/4 mucositis in precancerous tissue as a function of time post-treatment. Error bars are ± SE.

tissue around treated tumors (*14, 18, 26*), we devised "Sequential" BNCT, a new approach to BNCT, to optimize tumor response without exceeding the tolerance of precancerous tissue. Precancerous tissue was considered to tolerate treatment if mucositis resolved within the 28-day observation period. "Sequential" BNCT involved the application of BNCT mediated by BPA followed 24 or 48 h later by BNCT mediated by GB-10. The present study unequivocally demonstrated a therapeutic advantage for "Sequential" BNCT over the total physical dose-matched single application of (BPA + GB-10)-BNCT by producing enhancement of tumor response with no additional mucositis in the dose-limiting precancerous tissue surrounding the treated tumors. The present study did not explore the potential effects of sequential applications using the same boron agent and only provides evidence for an improved therapeutic effect of BPA-BNCT followed by GB-10-BNCT compared to a single application of (BPA + GB-10)-BNCT. The sequential application of GB-10-BNCT followed by BPA-BNCT could be less effective based on the hypothesis that the vascular targeting effects of GB-10-BNCT (*18*) could impair the subsequent delivery of BPA. Sequential applications of BPA-BNCT would be interesting to evaluate in terms of tumor control but might pose a concern because of the known dose-limiting mucositis of BPA-BNCT in this model (*18*).

The "Sequential" BNCT and single application BNCT protocols were matched in terms of total physical dose prescribed to tumor. However, the effective doses could be different. The boron concentrations in tumor are variable and heterogeneous. The "Sequential" BNCT and the single application BNCT protocols differed in the total γ-ray dose (0.90 Gy and 0.71 Gy, respectively) and nitrogen capture proton dose (0.72 Gy and 0.57 Gy, respectively) (Table 2). The biologically effective doses will depend on the relative biological effectiveness (RBE) and compound biological effectiveness (CBE) (*2*) for the different dose components in this model, and they are currently being determined.

Both "Sequential" BNCT protocols exhibited similar high overall tumor responses of 91–95%, while the overall response induced by the tumor dose-matched single application of (BPA + GB-10)-BNCT was significantly lower (75%). The complete tumor response rate was also significantly higher for the "Sequential" protocols than for the single application of (BPA + GB-10)-BNCT. Both "Sequential" BNCT protocols and the single application of (BPA + GB-10)-BNCT induced reversible mucositis in precancerous tissue; the rates of Grade 3/4 mucositis did not differ significantly. In terms of toxicity to precancerous tissue, there were no undue adverse effects associated with the improved tumor response from the "Sequential" BNCT protocols.

The "Sequential" modality was devised based on notions of BNCT radiobiology contributed by previous studies by our group and others (*e.g. 2, 18*). The therapeutic advantage of "Sequential" BNCT could be ascribed to factors such as those described below.

"Sequential" BNCT involves the use of two boron agents with different properties and complementary mechanisms of action, conceivably contributing to a more homogeneous, therapeutically successful targeting of heterogeneous tumor cells populations (*18, 19*). The brief interval between applications would favor targeting with GB-10. It is known that interstitial fluid pressure (IFP) is elevated in most human and experimental tumors (*34, 35*) mainly as a result of unregulated angiogenesis. The resulting hyperpermeable blood vessels lead to an initial net efflux of fluid into the tumor interstitium. Since solid tumors usually lack functional lymphatic vessels capable of maintaining fluid homeostasis, both hydrostatic and oncotic pressures become almost equal in the

intravascular and extravascular spaces, thus hindering convective fluid transport. In addition, tumor cells proliferate in a confined space and tumor interstitium consists of dense collagen fibers and increased inflammatory components. Elevated tumor IFP is partly responsible for the poor distribution of blood-borne therapeutic agents (*34, 36*). A decrease in IFP has been reported to occur shortly after irradiation [e.g., 1–2 days after fractionated or single doses greater than 10 Gy of photons (*34*)]. A reduction in IFP improves the uptake of therapeutic agents (*e.g. 37*). In addition, the induction of void space by cancer cell death enhances the intratumoral delivery of therapeutic agents (*38*). The therapeutic effect of the first application of BPA-BNCT at a total physical tumor dose of approximately 4 Gy (unweighted for relative biological effectiveness) could conceivably reduce IFP and induce cancer cell death, favoring the penetration and distribution of GB-10. Thus GB-10 would have a better chance of targeting a tumor when it is administered as part of the ''Sequential'' protocol than when it is administered with BPA in the single application protocol. Microenvironmental changes in the tumor after the first cycle of therapy might lead to changes in boron microdistribution in the second cycle. Changes in boron microdistribution were not seen in the ICP-OES and ICP-MS gross boron measurements or in the preliminary neutron autoradiography studies and must be investigated in future studies using appropriate methods such as neutron autoradiography (*6*) and quantitative secondary ion mass spectrometry (SIMS) (*39*). The SIMS technique is uniquely suited for microlocalization studies of two boron compounds used alone or in combination and would contribute to elucidating the cellular and subcellular mechanisms that determine the responses of tumor and precancerous tissue.

For the ''Sequential'' BNCT protocols, the incidence of complete response was greater for the small tumors than for the medium and large tumors. This finding could be attributed to the fact that IFP increases with the size of the tumor (*35*), conceivably impairing the distribution of boron compounds more in the larger tumors. The therapeutic benefit of the ''Sequential'' BNCT protocol therefore might be less robust for the larger tumors than for the smaller tumors.

Lengthening overall treatment time in conventional radiotherapy reduces normal tissue toxicity but also reduces tumor control probability (*40, 41*). In the case of BNCT, in which the radiation dose is composed of a combination of high- and low-LET radiation components, the brief interval (1 or 2 days) between treatments would produce little change in the repair of sublethal damage. However, this interval, which is short enough to preclude tumor cell repopulation (*42, 43*), could favor targeting of the tumor cells that were refractory to the first application. Both intervals employed here (1 or 2 days) seem to be equally effective in enhancing tumor response.

Regarding the finding that Seq-BNCT improved tumor control over (BPA + GB-10)-BNCT with no additional toxicity, it is known that a faster rate of basal cell proliferation makes tissues more prone to develop mucositis (*33*). The reduction in DNA synthesis induced by BPA-BNCT described previously in precancerous hamster cheek pouch tissue (*44*) could therefore render the tissue exposed to the second application of BNCT (GB-10-BNCT) less or at worst equally liable to develop mucositis than if the total dose is delivered in a single application. These effects would apply mainly to the low-LET dose components of BNCT. Because mucositis is a multistage process initiated by mucosal injury and associated to an increased production of inflammatory cytokines that cause direct mucosal damage and initiate positive feedback loops (*45*), the 24- or 48-h interval between BNCT applications might conceivably allow the inflammatory process to partially subside before the second dose is delivered, precluding the exacerbation of mucositis. Although differences undoubtedly exist between the precancerous tissue in the hamster cheek pouch model and precancerous tissue around SCC in human subjects (*18*), a therapeutic strategy that reduces precancerous tissue toxicity in the experimental model could also conceivably contribute to reducing toxicity in human oral mucosa.

The sequential application of BPA-BNCT followed by GB-10-BNCT, as opposed to a single application of (BPA + GB-10)-BNCT, would allow for modulation of each application with appropriate methods tailored for each boron carrier. An additional asset of ''Sequential'' BNCT is that it employs BPA and GB-10, both of which are approved for use in human subjects. Furthermore, given the similarity between GB-10 and BSH in terms of biodistribution and lack of tumor selectivity (*18*) and the clinical interest in BSH alone or combined with BPA (e.g. *46*), it would be of interest to explore the efficacy of ''Sequential'' BNCT employing BSH instead of GB-10.

Within the context of recent (ongoing) BNCT clinical trials for recurrent head and neck malignancies that showed encouraging tumor control associated with dose-limiting mucositis (*8, 47*), the search for novel BNCT strategies that improve tumor control at no extra cost in terms of mucositis is particularly relevant. We can conclude based on the observations reported here that ''Sequential'' BNCT may be a clinically promising BNCT modality for head and neck cancer, one that enhances tumor response at no extra cost in terms of toxicity in the dose-limiting precancerous tissue and is thus deserving of further investigation.

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