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Tumor Blood Vessel "Normalization" Improves the Therapeutic Efficacy of Boron Neutron Capture Therapy (BNCT) in Experimental Oral Cancer

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We previously demonstrated the efficacy of BNCT mediated by boronophenylalanine (BPA) to treat tumors in a hamster cheek pouch model of oral cancer with no normal tissue radiotoxicity and moderate, albeit reversible, mucositis in precancerous tissue around treated tumors. It is known that boron targeting of the largest possible proportion of tumor cells contributes to the success of BNCT and that tumor blood vessel normalization improves drug delivery to the tumor. Within this context, the aim of the present study was to evaluate the effect of blood vessel normalization on the therapeutic efficacy and potential radiotoxicity of BNCT in the hamster cheek pouch model of oral cancer. Blood vessel normalization was induced by two doses of thalidomide in tumor-bearing hamsters on 2 consecutive days. All studies in thalidomide-treated animals were performed 48 h after the first dose of thalidomide, previously established as the window of normalization. Biodistribution studies were performed with BPA at a dose of 15.5 mg ^{10}B/kg in thalidomide-treated (Th+) and untreated (Th–) tumor-bearing hamsters. The effect of blood vessel normalization prior to BPA administration on the efficacy of BNCT was assessed in *in vivo* BNCT studies at the RA-3 Nuclear Reactor in tumor-bearing hamsters. Group I was treated with BPA-BNCT after treatment with thalidomide (Th+ BPA-BNCT). Group II was treated with BPA-BNCT alone (Th– BPA-BNCT). Group III was treated with the beam only after treatment with thalidomide (Th+ BO), and Group IV was treated with the beam only (Th– BO). Groups I and II were given the same dose of BPA (15.5 mg ^{10}B/kg), and all groups (I–IV) were exposed to the same neutron fluence. Two additional groups were treated with the beam only at a higher dose to exacerbate mucositis in precancerous tissue and to explore the potential direct protective effect of thalidomide on radiation-induced mucositis in a scenario of more severe toxicity, i.e. Group V (Th+ hdBO) and Group VI (Th– hdBO). The animals were followed for 28 days. Biodistribution studies revealed no statistically significant differences in gross boron content between Th+ and Th– animals. Overall tumor control (complete response + partial response) at 28 days post-treatment was significantly higher for Group I (Th+ BPA-BNCT) than for Group II (Th– BPA-BNCT): 84 ± 3% compared to 67 ± 5%. Pretreatment with thalidomide did not induce statistically significant changes in overall tumor control induced by the beam only, i.e. 15 ± 5% in Group III (Th+ BO) and 18 ± 5% in Group IV (Th– BO), or in overall tumor control induced by the high-dose beam only, i.e. 60 ± 7% in Group V (Th+ hdBO) and 47 ± 10% in Group VI (Th– hdBO). BPA-BNCT alone (Group II) induced mucositis in precancerous tissue that reached Grades 3–4 in 80% of the animals, whereas pretreatment with thalidomide (Group I) prevented mucositis Grades 3 and 4 completely. Beam-only Group III (Th+ BO) exhibited only Grade 1 mucositis in precancerous tissue, whereas 17% of the animals in beam-only Group IV (Th– BO) reached Grade 2 mucositis. High-dose beam-only group V (Th+ hdBO) exhibited only Grade 2 mucositis, whereas high-dose beam-only group VI (Th– hdBO) reached Grade 3 mucositis in 83% of the animals. In all cases mucositis in precancerous tissue was reversible. No normal tissue radiotoxicity was observed with any of the protocols. Pretreatment with thalidomide enhanced the therapeutic efficacy of BNCT and reduced precancerous tissue toxicity.

INTRODUCTION

Boron neutron capture therapy (BNCT) is a binary cancer treatment modality that combines irradiation with a thermal or epithermal neutron beam with boron-10 (^{10}B) carriers that are taken up preferentially by neoplastic cells. The high-linear energy transfer α particles and recoiling ^{7}Li nuclei emitted during the ^{10}B(n,α)^{7}Li reaction have a range of 5–9

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μm in tissue and are known to have a high relative biological effectiveness (RBE). In this way, BNCT would potentially target tumor tissue selectively, mostly sparing normal tissue (*1, 2*).

Clinical trials of BNCT for the treatment of glioblastoma multiforme and/or 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, Europe, Japan, Taiwan and the U.S. (e.g. *3–10*). To date, the clinical results have shown a potential therapeutic advantage for this technique but undoubtedly leave room for improvement. Contributory translational studies have been carried out employing a variety of experimental models based on the implantation of tumor cells in normal tissue (e.g. *11*).

To explore new applications of BNCT and to study the radiobiology of BNCT to improve its therapeutic efficacy, we previously proposed and validated the use of the hamster cheek pouch model of oral cancer for BNCT studies (*12, 13*). Despite considerable advances in the understanding of the etiology of head and neck malignancies and the use of new treatment modalities, their management continues to be difficult. Squamous cell carcinoma (SCC) of the head and neck region is the sixth most common cause of cancer deaths worldwide, and the incidence is rising rapidly in developing countries. The relatively poor overall 5-year survival rate for malignancies of the oral cavity is estimated to range between 58.3% and 63% (*14*). Within this context, and in view of the fact that radical surgery causes large tissue defects (*15*), there is a need for more effective and less toxic therapies that can discriminate between malignant and normal cells. Studies in appropriate experimental models are pivotal to progress in this field. The hamster cheek pouch model of carcinogenesis is widely accepted as a model of oral cancer (*16, 17*). Carcinogenesis protocols induce premalignant and malignant changes that closely resemble spontaneous human oral mucosa lesions (*18*). In addition, the hamster cheek pouch model of oral cancer possesses an 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. Conversely, the tumor models classically employed in BNCT small-animal studies are based on the growth of implanted cancer cells in healthy tissue (e.g. *11*). In the hamster cheek pouch, carcinogenesis protocols lead to the development of what has been called, globally, ''precancerous tissue'' (e.g. *12*) or, more recently, ''tissue with potentially malignant disorders (PMD)'' (*19*), from which tumors arise. Thus this mode of tumor induction provides a tumor model surrounded by precancerous tissue. The possibility of studying precancerous tissue in addition to tumor and normal tissue is clinically relevant in terms of its role as a potentially dose-limiting tissue.

In previous studies we demonstrated that potentially therapeutic boron concentrations could be delivered to hamster cheek pouch tumors employing BPA as the boron delivery agent (*12*). We then performed *in vivo* BNCT studies at the RA-6 Nuclear Reactor and provided the first evidence of the therapeutic success of BNCT mediated by BPA to treat oral cancer in an experimental model with no damage to normal tissue and moderate, albeit reversible, mucositis in precancerous tissue (*13*). In a preclinical scenario, the potential of BPA-BNCT to treat spontaneous SCC was demonstrated in felines (*20, 21*). Subsequent studies (*22*) at the RA-3 Nuclear Reactor biomedical thermal facility (*23*) also showed marked therapeutic efficacy of BNCT in the hamster cheek pouch oral cancer model but prompted optimization focused on increasing tumor control and/or reducing toxicity in the dose-limiting precancerous tissue. Dose escalation to improve tumor control is limited by mucositis in precancerous tissue in the hamster cheek pouch oral cancer model (*13, 22, 24, 25*). In a clinical scenario, oral mucositis limits the dose that can be administered with BNCT to head and neck and brain tumors (*2, 6, 26*) and is a frequent dose-limiting side effect during conventional radiotherapy for advanced head and neck tumors, affecting approximately 80% of the patients (*27, 28*). Despite its incidence and clinical relevance, no effective way to prevent or treat mucositis is currently available (*28, 29*). Within this context, BNCT protocols that reduce mucositis are more likely to deliver therapeutically useful doses to tumor without exceeding normal and precancerous tissue tolerance.

Targeting of the largest possible proportion of tumor cells contributes to the success of BNCT in particular and of oncological therapies in general (*24, 25*). Those tumor cell populations that are poorly loaded with boron will be significantly less responsive or altogether refractory to BNCT. Within this context, optimization of the delivery of boron compounds to neoplastic cells will contribute to the therapeutic efficacy of BNCT. It is well known that tumor vessels are characteristically dilated, saccular and tortuous and exhibit large interendothelial cell junctions, increased numbers of fenestrations, and lack of normal basement membrane. Leaky, hyperpermeable blood vessels lead to an elevation in interstitial fluid pressure. In addition, proliferating tumor cells exert compressive forces on blood vessels (*30*). The abnormal structure and function of tumor blood vessels compromise blood flow and hinder effective convective fluid transport, resulting in the impaired distribution of blood-borne therapeutic agents (*31, 32*). Tumor blood vessel normalization by tailored administration of antiangiogenic agents that downregulate vascular endothelial growth factor (VEGF) (overexpressed in the majority of solid tumors) would lead to less leaky, less dilated and less tortuous vessels, decreased interstitial fluid pressure, increased tumor oxygenation, and improved penetration of drugs in tumors (*32, 33*). Within this context, reversible tumor blood vessel normalization prior to boron

compound administration would conceivably improve boron targeting and BNCT efficacy. In view of the fact that the anti-angiogenic monoclonal antibodies employed to induce blood vessel normalization in human subjects, rats and mice (e.g. *32*) cannot be used in hamsters due to lack of cross-antigenicity, we previously developed a technique to normalize aberrant blood vessels in the hamster cheek pouch oral cancer model employing thalidomide as an antiangiogenic drug (*34*), thus rendering the hamster cheek pouch oral cancer model amenable to blood vessel normalization studies.

The aim of the present study was to assess the effect of tumor blood vessel normalization prior to administration of BPA on the therapeutic efficacy and potential toxicity of BNCT mediated by BPA in the hamster cheek pouch oral cancer model.

MATERIALS AND METHODS

Tumor Induction

Tumors were induced in the right cheek pouch of noninbred young (6 weeks old) Syrian hamsters by topical application of 0.5% of the complete carcinogen dimethyl-1,2-benzanthracene (DMBA) in mineral oil twice a week for 12 weeks in keeping with a standard hamster cheek pouch carcinogenesis protocol (*35*) modified as described previously (e.g. *25*). The treated pouch was periodically everted under light intraperitoneal (i.p.) ketamine (70 mg/kg body weight)-xylazine (10.5 mg/kg body weight) anesthesia and examined to monitor tumor development. Once the exophytic tumors, i.e. squamous cell carcinomas, developed and reached a diameter of approximately 3–5 mm, the animals were used for biodistribution studies and *in vivo* BNCT studies. A variable number of tumors developed in each cancerized pouch. Experimental protocols were approved by the institutional Ethical Committee.

Blood Vessel Normalization

In keeping with a previously established technique (*34*), two doses of 200 mg thalidomide/kg body weight in dimethyl sulfoxide (DMSO) (112 mg thalidomide/ml DMSO) were administered intraperitoneally to tumor-bearing hamsters on 2 consecutive days. All experiments in thalidomide-treated animals were performed 48 h after the first injection of the drug, previously established as the window of normalization. Thalidomide was a generous gift of Triquim S.A. and Laboratorio Lazar (Argentina).

Biodistribution Studies

BPA biodistribution studies were performed in a group of three hamsters bearing a total of seven tumors, pretreated with thalidomide (Th+), and in a group of four hamsters bearing a total of eight tumors, not pretreated with thalidomide (Th–). In thalidomide-treated animals, BPA was administered intraperitoneally at a dose 15.5 mg ^{10}B/kg body weight 48 h after the first injection of the drug. Forty-eight hours after the first dose of thalidomide was previously established as the window of normalization (*34*). Samples of blood, tumor, precancerous pouch tissue, normal pouch tissue and liver (as a reference) were taken 3 h postadministration of BPA in both groups. All the samples were weighed immediately. Until use, tissue samples were stored at −20°C, and blood samples were stored with EDTA 5% v/v at 4°C. The samples were processed for gross boron measurement by inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC2, Perkin Elmer). Tissue samples (30–50 mg) and blood samples (200–300 μl) were digested in 15-ml Falcon tubes for 1 h at 100°C in 0.25 ml of a 1:1 mixture of ultrapure concentrated sulfuric acid (J. T. Baker, Phillipsburg, NJ) and sub-boiling nitric acid distilled from 65% nitric acid (p.a., Carlo Erba, Milan, Italy). Once the digestion process was complete, the mixture was allowed to cool and Milli-Q water was added to bring the final volume to 10 ml. The digested samples were stored at room temperature for a maximum of 3 days prior to measurement. All the digested samples were vortexed immediately prior to preparation for actual measurement. Approximately 1 ml of the digested tissue sample or 0.2 ml of the digested blood sample (depending on estimated boron content) was placed in a new Falcon tube and mixed with 0.20 ml of a 1:1 mixture of ultrapure concentrated sulfuric acid and sub-boiling nitric acid; 0.25 ml of ^{6}Li (1 ppm) was added as an internal standard. Milli-Q water was added to bring the final volume to 10 ml. All the prepared samples were vortexed immediately prior to measurement. Standard solutions of boric acid (enriched to 99.8% in ^{10}B) were used to prepare a calibration line each day of operation.

In Vivo BNCT

Irradiations were performed at a 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 (*23*). 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 as described previously (e.g. *22, 25*). Physical dosimetry data corresponding to the irradiation system have been reported previously (*22*). Briefly, the thermal neutron flux is about 9.1×10^{9} n cm^{-2} s^{-1} in the outermost position on the pouch shelf and 7.7×10^{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 container 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}.

Group I (7 hamsters) bearing a total of 116 tumors was treated with thalidomide followed by BNCT mediated by BPA 48 h after the first dose of thalidomide (Th+ BPA-BNCT). Group II (5 hamsters) bearing a total of 100 tumors was treated only with BNCT mediated by BPA (Th– BPA-BNCT). A total absorbed dose of 4 Gy was prescribed to the tumor. Group III (6 hamsters) bearing a total of 54 tumors was treated with thalidomide followed by the beam only 48 h after the first dose of thalidomide (Th+ BO). Group IV (6 hamsters) bearing a total of 50 tumors was treated the with beam only (Th– BO). Neutron irradiation was performed 48 h after the first dose of thalidomide in Th+ animals, the time previously established as the normalization window (*34*). The beam-only irradiations [neutron irradiation with no prior administration of boron compound(s)] were performed to evaluate the effect of the background dose (i.e. γ rays and ^{14}N thermal neutron capture-induced protons) of the BNCT treatments and were matched for thermal neutron fluence with the corresponding BNCT treatments, resulting in a total absorbed dose to tumor of 0.89 ± 0.02 Gy. In this way, all animals (Groups I–IV) were exposed to the same thermal neutron fluence (1.9×10^{12} n cm^{-2}). For BPA-BNCT, BPA (0.14 *M*) was administered i.p. at a dose of 15.5 mg ^{10}B/kg body weight. The animals were irradiated 3 h after administration of BPA under i.p. ketamine (140 mg/kg body weight)–xylazine (21 mg/kg

TABLE 1
Boron Concentrations (mean ± SD)

	Th+		Th–	
	n	ppm[a]	*n*	ppm[a]
Tumor	7	20 ± 8	8	24 ± 6
Precancerous	3	14 ± 3	4	13 ± 2
Normal pouch	3	15 ± 4	4	15 ± 1
Liver	3	7 ± 2	4	8 ± 1
Blood	5	5 ± 2	4	9 ± 1

[a] 3 h after administration of BPA at 15.5 mg ^{10}B/kg body weight bolus i.p.

body weight) anesthesia. The BPA dose and the time postadministration used for neutron irradiation were selected on the basis of previous studies (*12, 13*). In view of the finding (described in the Results section) that pretreatment with thalidomide led to a reduction in BPA-BNCT-induced precancerous tissue mucositis, two additional groups irradiated with the beam only were included solely to explore the potential direct protective effect of thalidomide on radiation-induced mucositis (independent of its potential action on BPA delivery) in a scenario of more severe toxicity. Group V (6 hamsters) bearing a total of 48 tumors was treated with thalidomide followed by the beam only at a higher total absorbed dose to tumor (5.6 ± 0.5 Gy) (Th+ hdBO) and Group VI (6 hamsters) bearing a total of 53 tumors was treated similarly with no pretreatment with thalidomide (Th– hdBO).

Dosimetric calculations for the BPA-BNCT protocols were based on boron biodistribution data (see Results section).

Follow-up

To evaluate toxicity, clinical signs and body weight of all the animals were monitored daily for the first week and then twice a week for the remaining follow-up period. The tumor and precancerous tissue responses were assessed by visual inspection and tumor volume assay pretreatment and at 1, 7, 14, 21 and 28 days post-treatment. Tumor volume was determined by external caliper measurement of the three largest orthogonal diameters (d) and expressed in mm^3 as $d_1 \times d_2 \times d_3$ in keeping with previous studies (*13, 24*). A reduction from initial tumor volume was considered as partial response (PR) as defined in previous studies (*13, 24*). A reduction to ≤50% of the initial tumor volume was referred to as partial response$_{0.5}$ (PR$_{0.5}$) (*25*). Complete tumor response (CR) was defined as disappearance of the tumor on visual inspection. Overall tumor response (OR) was defined as PR + CR in keeping with previous studies (*13, 24*). Tumor-bearing animals were monitored 1, 7, 14, 21 and 28 days post-treatment. The severity of mucositis was evaluated semi-quantitatively in dose-limiting precancerous tissue according to an oral mucositis scale based on macroscopic features, adapted for carcinogen-treated hamster cheek pouch from the WHO classification for oral mucositis in human subjects (*36*) and the six-point grading system for normal hamster cheek pouches of Sonis *et al*. (*37*), 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.

The statistical significance of the differences in gross boron content was evaluated by Student's *t* test. When pertinent, statistical analysis of differences in tumor response and precancerous tissue mucositis was performed using Fisher's exact test. Statistical significance was set at $P = 0.05$.

RESULTS

Gross boron concentration data are summarized in Table 1. Boron concentration in tumors fell within a therapeutically useful range (*24*). No statistically significant differences were found in gross boron content between Th+ and Th– animals. Table 2 presents the prescribed absorbed doses from the different radiation components, the corresponding irradiation times and thermal neutron fluence, and the corresponding total absorbed prescribed doses for the different treatment protocols.

Tumor response was evaluated at 28 days post-treatment considering three arbitrary tumor sizes (small: <10 mm^3, medium: 10–100 mm^3, large: >100 mm^3) defined to categorize tumor size at the time of irradiation and evaluate potential differential response according to size (*38, 39*). Tumor response for Group I (Th+ BPA-BNCT) and Group II (Th– BPA-BNCT) are presented in Table 3. Overall tumor response (OR) for all tumor sizes taken together was

TABLE 2
Absorbed Doses (Gy) for the Different Experimental Protocols

Protocol	Tissue	Irradiation time (min)	Fluence (n cm^{-2})	Total γ-ray dose (Gy)	Induced protons (N14) (Gy)	Boron dose (Gy)	Total without boron (Gy)	Total dose (Gy)
Th+ BPA-BNCT	Tumor	4.1 ± 0.2	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	3 ± 1	0.9 ± 0.1	4 ± 1
	Precancerous	4.1 ± 0.2	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	2.0 ± 0.5	0.9 ± 0.1	2.9 ± 0.5
	Normal pouch	4.1 ± 0.2	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	2.1 ± 0.6	0.9 ± 0.1	3.0 ± 0.6
BPA-BNCT	Tumor	4.2 ± 0.3	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	3.4 ± 0.9	0.9 ± 0.1	4.3 ± 0.9
	Precancerous	4.2 ± 0.3	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	1.8 ± 0.3	0.9 ± 0.1	2.7 ± 0.3
	Normal pouch	4.2 ± 0.3	(1.9 ± 0.4) × 10^{12}	0.49 ± 0.01	0.40 ± 0.09	2.1 ± 0.2	0.9 ± 0.1	3.0 ± 0.2
Beam only (Th+ and Th–)	Tumor	4.1 ± 0.3	(1.9 ± 0.4) × 10^{12}	0.44 ± 0.01	0.40 ± 0.09	0.0 ± 0.0[a]	0.8 ± 0.1	0.8 ± 0.1
Th+ hdBO	Tumor	28.9 ± 0.1	(1.3 ± 0.3) × 10^{13}	2.85 ± 0.04	2.8 ± 0.5	0.0 ± 0.0[a]	5.6 ± 0.5	5.6 ± 0.5
Th− hdBO	Tumor	29.0 ± 0.2	(1.3 ± 0.3) × 10^{13}	2.93 ± 0.04	2.7 ± 0.5	0.0 ± 0.0[a]	5.6 ± 0.5	5.6 ± 0.5

[a] Boron concentration in tissues of animals not injected with BPA was generally below the detection limit. The maximum boron concentration value recorded was 0.4 ± 0.1 ppm. Even in this case the variation in total absorbed dose would fall within the uncertainty of dose determination.

TABLE 3
Tumor Response (% ± SE): BNCT Protocols

Tumors	*n*	Complete response	[a] Partial response	[b] Partial response$_{0.5}$ as reduction to ≤50% of initial volume	No response	Overall response (partial + complete response)
Animals treated with thalidomide before BPA-BNCT (Th+ BPA-BNCT) (7 hamsters) BPA (15.5 mg ^{10}B/kg body weight)						
Total	116	56 ± 5	28 ± 4	20 ± 4	16 ± 3	84 ± 3
Large: >100 mm^3	7	14 ± 13	57 ± 19	43 ± 19	29 ± 17	71 ± 17
Medium: 10–100 mm^3	32	25 ± 8	59 ± 9	41 ± 9	16 ± 6	84 ± 6
Small: <10 mm^3	77	73 ± 5	13 ± 4	9 ± 3	14 ± 4	86 ± 4
Animals not treated with thalidomide before BPA-BNCT (Th– BPA-BNCT) (5 hamsters) BPA (15.5 mg 10B/kg body weight)						
Total	100	43 ± 5	24 ± 4	13 ± 3	33 ± 5	67 ± 5
Large: >100 mm^3	4	0	50 ± 25	25 ± 22	50 ± 25	50 ± 25
Medium: 10–100 mm^3	17	24 ± 10	47 ± 12	35 ± 12	29 ± 11	71 ± 11
Small: <10 mm^3	79	49 ± 6	18 ± 4	8 ± 3	33 ± 5	67 ± 5

Notes. *n*: number of tumors. Category [b] is included in category [a], making the sum of percentages of a single row larger than 100%.

TABLE 4
Tumor Response (% ± SE): Beam Only Protocols

Tumors	*n*	Complete response	[a] Partial response	[b] Partial response$_{0.5}$ as reduction to ≤50% initial volume	No response	Overall response (partial + complete response)
Animals treated with thalidomide before beam only (Th+ BO) (6 hamsters)						
Total	54	0	15 ± 5	4 ± 3	85 ± 5	15 ± 5
Large: >100 mm^3	2	0	0	0	100 ± 0	0
Medium: 10–100 mm^3	9	0	22 ± 14	0	78 ± 14	22 ± 14
Small <10 mm^3	43	-	14 ± 5	5 ± 3	86 ± 5	14 ± 5
Animals not treated with thalidomide before beam only (Th– BO) (6 hamsters)						
Total	50	-	18 ± 5	6 ± 3	82 ± 5	18 ± 5
Large: >100 mm^3	2	0	0	0	100 ± 0	0
Medium: 10–100 mm^3	10	-	20 ± 13	0	80 ± 13	20 ± 13
Small <10 mm^3	38	0	18 ± 6	8 ± 4	82 ± 6	18 ± 6

Notes. *n*: number of tumors. Category [b] is included in category [a], making the sum of percentages of a single row larger than 100%.

significantly higher for animals pretreated with thalidomide prior to BPA-BNCT (Group I) than for animals treated with BPA-BNCT alone (Group II), 84 ± 3% compared to 67 ± 5% ($P = 0.006$). Considering stratification for tumor size at the time of irradiation, overall tumor response was higher in Th+ BPA-BNCT animals than in Th– BPA-BNCT animals for large tumors (71 ± 17% and 50 ± 25%, respectively), medium tumors (84 ± 6% and 71 ± 11%, respectively), and small tumors (86 ± 4% and 67 ± 5%, respectively). These differences reached statistical significance for small tumors ($P = 0.008$). Complete tumor response for all tumor sizes taken together was higher for thalidomide-treated animals than for animals not treated with thalidomide (56 ± 5% and 43 ± 5%, respectively). This difference reached statistical significance for small tumors ($P = 0.003$). It is noteworthy that no statistically significant differences were found between the PR data and PR$_{0.5}$ data. Only PR data were considered in the analysis for the sake of simplicity. The incidence of complete response was higher for the small tumors than for the medium and large tumors in Groups I and II. This difference reached statistical significance in the case of Group I ($P \leq 0.004$). Complete response in large tumors is characteristically difficult to achieve (*38, 39*) and compelling to analyze. However, the relative scarcity of overly large tumors (>100 mm^3) in the hamster cheek pouch model of oral cancer complicates the analysis of response in this particular group of tumors.

Tumor responses for Group III (Th+ BO) and Group IV (Th– BO) are presented in Table 4. No significant differences in tumor response were observed as a result of pretreatment with thalidomide in beam-only treated animals.

Additionally, and regardless of pretreatment with thalidomide, highly statistically significant differences in overall tumor response were found between the groups treated with BPA-BNCT and those treated with the beam only (Tables 3 and 4), i.e. Group I (84 ± 3%) compared to Group III (15 ± 5%), $P = 0.012$; Group I (84 ± 3%) compared to Group IV (18 ± 5%), $P = 0.0001$; Group II (67 ± 5%) compared to Group III (15 ± 5%), $P = 0.0001$; Group II (67 ± 5%) compared to Group IV (18 ± 5%), $P = 0.0001$.

Mucositis in precancerous tissue peaked at 7–10 days after BPA-BNCT and had decreased by 2–3 weeks post-treatment. Considering overall incidence of mucositis in the study period, pretreatment with thalidomide reduced the

TABLE 5
Severity of Mucositis in Precancerous Tissue: Percentage Incidence of Grade 2 to Grade 4 Mucositis Considering the Whole Study Period

Treatment protocol	Total number of hamsters	Grade 2	Grade 3	Grade 4	Grade 3–4
Th+ BPA-BNCT (Group I)	7	86	0	0	0
Th− BPA-BNCT (Group II)	5	80	60	20	80
Th+ BO (Group III)	6	0	0	0	0
Th− BO (Group IV)	6	17	0	0	0
Th+ hdBO (Group V)	6	100	0	0	0
Th− hdBO (Group VI)	6	67	83	0	83

Note. The values indicate the percentages of animals whose precancerous tissue exhibited the mucositis grade shown in each column at one or more of the follow-up times (all animals exhibited at least Grade 1 mucositis during follow-up).

incidence of Grade 3–4 mucositis from 80% in Group II to 0% in Group I (Table 5). Mucositis did not exceed Grade 2 in any of the animals treated with the beam only (Group III). Pretreatment with thalidomide reduced the incidence of Grade 2 mucositis in animals treated with the beam only from 17% to 0%. This finding suggested a protective effect of pretreatment with thalidomide on precancerous tissue mucositis. In animals treated with the beam only, the observed effects were direct, i.e. independent of a potential action on BPA delivery. However, since mucositis was so mild in animals treated with the beam only at the fluence-matched dose (Table 2), it was decided to further explore this effect at a higher beam-only dose that would exacerbate mucositis in precancerous tissue as described in the Materials and Methods section.

Tumor responses for Group V (Th+ hdBO) and Group VI (Th– hdBO) are presented in Table 6. Pretreatment with thalidomide did not induce statistically significant differences in overall tumor response (Th+ hdBO: 60 ± 7% compared to Th– hdBO: 47 ± 10%) or in overall complete response (Th+ hdBO: 27 ± 6% compared to Th– hdBO: 13 ± 6%). However, remarkably, pretreatment with thalidomide induced a marked protective effect on precancerous tissue mucositis. Whereas 83% of the animals in Group VI (Th– hdBO) exhibited Grade 3 mucositis, none of the animals in group V (Th+ hdBO) reached Grade 3 mucositis (Table 5).

No normal tissue radiotoxicity was observed with any of the protocols. No changes were observed in the health status of the treated animals. Body weight values oscillated slightly over the post-treatment period evaluated, with variations (gain or loss) that did not exceed on average 10% of the initial weight and that were considered non-contributory. As reported previously (*34*), treatment with thalidomide induced a mostly reversible sedative effect that was enhanced by anesthesia.

DISCUSSION

Although we previously demonstrated the therapeutic success of BNCT mediated by BPA in the hamster cheek pouch oral cancer model (*13, 22*), we face the ongoing challenge of optimizing this technique, employing approaches that would conceivably also be useful in other experimental models and, eventually, in a clinical scenario. Within this context, we have successfully employed strategies such as the combined use of boron compounds to improve boron targeting homogeneity (*24*) and have

TABLE 6
Tumor Response (% ± SE): High-Dose Beam-Only Protocols

Tumors	*n*	Complete response	[a] Partial response	[b] Partial response$_{0.5}$ as reduction to ≤50% initial volume	No response	Overall response (partial + complete response)
Animals treated with thalidomide before high-dose beam only (Th+ hdBO) (6 hamsters)						
Total	48	27 ± 6	33 ± 7	17 ± 5	40 ± 7	60 ± 7
Large: >100 mm^3	0					
Medium: 10–100 mm^3	7	14 ± 13	71 ± 17	71 ± 17	14 ± 13	86 ± 13
Small: <10 mm^3	41	29 ± 8	27 ± 7	7 ± 4	44 ± 8	56 ± 8
Animals not treated with thalidomide before high-dose beam only (Th– hdBO) (6 hamsters)						
Total	53	13 ± 6	34 ± 9	17 ± 5	53 ± 10	47 ± 10
Large: >100 mm^3	2	0	50 ± 35	50 ± 35	50 ± 35	50 ± 35
Medium: 10–100 mm^3	11	0	45 ± 22	27 ± 13	55 ± 22	45 ± 22
Small: <10 mm^3	40	18 ± 9	30 ± 10	13 ± 5	52 ± 11	48 ± 11

Notes. *n*: number of tumors. Category [b] is included in category [a], making the sum of percentages of a single row larger than 100%.

recently reported the improvement in tumor response at no cost in terms of toxicity in dose-limiting precancerous tissue employing ''Sequential'' BNCT (*25*). Given that optimizing the targeting of boron compounds will contribute to the efficacy of BNCT (e.g. *24, 40*), we recently developed a technique to induce aberrant blood vessel normalization in the hamster cheek pouch oral cancer model employing thalidomide (*34*). Based on the knowledge that blood vessel normalization improves drug delivery and distribution to tumor cells (*30*), our working hypothesis was that administration of BPA in the ''window of normalization'' (Fig. 1) would improve boron targeting of tumor cells and would thus enhance tumor control. In this sense it was essential to first determine the ''window of normalization'' (*34*) because it is known from chemotherapy studies that no therapeutic benefit is obtained when the drug is administered outside this time window (*41*). We thus assessed the effect of aberrant blood vessel normalization on the therapeutic success and toxicity of BNCT in the hamster cheek pouch oral cancer model, administering BPA in the predetermined window of normalization.

The present study demonstrated that blood vessel normalization prior to administration of BPA enhanced tumor response to BPA-BNCT (Group I compared to Group II). The finding that pretreatment with thalidomide did not induce a significant change in tumor response in animals treated with the beam only (Group III compared to Group IV and Group V compared to Group VI) would suggest that the improvement in tumor response would be related to an effect on BPA delivery. The previously described improvement in blood flow and drug delivery by transient restoration of vascular function in tumors (e.g. *30*) led us to expect an increase in absolute boron content in tumors of animals treated with thalidomide prior to administration of BPA. However, gross boron measurements by ICP-MS failed to reveal statistically significant differences in gross boron content between Th+ and Th– animals. The previously described reduction in vessel permeability measured in terms of a decrease in Evans Blue extravasation in tumors with ''normalized'' blood vessels (*33, 34*) would appear to be counterintuitive. However, it is known that vascular hyperpermeability and leakiness in aberrant tumor blood vessels lead to interstitial hypertension and temporally and spatially heterogeneous blood flow. In this way drugs will accumulate in areas that already have a sufficient amount while inaccessible regions will continue to be inaccessible (*42*). In this sense, gross values of boron concentration tell us little about the adequacy and therapeutic usefulness of boron distribution, although gross boron values are used for dosimetric calculations (e.g. *2*). In fact, the primary aim of blood vessel normalization is not to increase total drug (and oxygen) uptake. Instead, it seeks to distribute drugs (and oxygen) effectively to a larger proportion of tumor cells by fixing the flawed delivery system (*32*). Within this context, it is not surprising that tumor gross boron values do not exhibit statistically

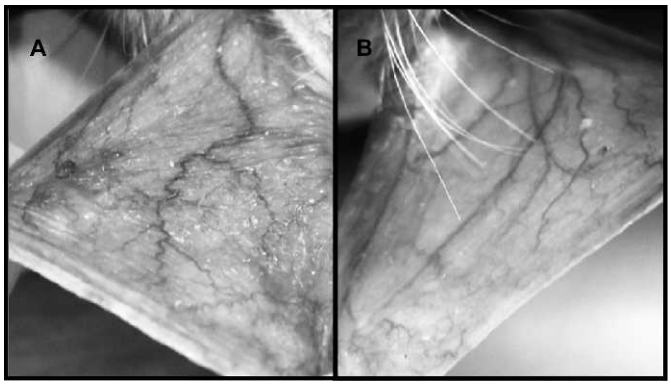

FIG. 1. Blood vessels seen *in vivo* by transparency in the precancerous tissue of an everted pouch before (panel A) and 48 h after (panel B) treatment with thalidomide. Thalidomide induced qualitative straightening and narrowing of the blood vessels, with a window of normalization at 48 h.

significant differences between Th+ and Th– animals. Preliminary α-particle spectrometry and neutron autoradiography studies in hamster cheek pouch tumors of Th+ and Th– animals showed that pretreatment with thalidomide did not increase the absolute boron content in oral tumors but improved boron targeting homogeneity (*43*). Although further studies are necessary to confirm these findings, they do suggest, within the context of the present study, that the improvement in tumor response elicited by aberrant blood vessel normalization prior to administration of BPA could be ascribed at least partially to an improvement in BPA distribution in tumors.

Improved oxygenation as a result of aberrant blood vessel normalization could conceivably play a role in increasing the sensitivity of tumors to the low-linear energy transfer (LET) radiation component in BNCT. However, the fact that no statistically significant difference in tumor response was found between Th+ and Th– groups treated with the beam only would suggest that the primary therapeutic benefit of blood vessel normalization would be associated to improved boron targeting. Thalidomide has been described as an immunomodulatory, anti-inflammatory and antiangiogenic drug. Its action as an inhibitor of angiogenesis is associated with its capacity to inhibit basic fibroblast growth factor (bFGF)- and vascular endothelium growth factor (VEGF)-induced angiogenesis (e.g. *44*). It has been shown that the short-term administration of antiangiogenic agents can prune inefficient vascular sprouts (*45*), yielding more efficient flow paths. Conversely, the long-term administration of thalidomide as an antiangiogenic drug could be expected to exert an antitumor effect (*46*). The fact that we found no statistically significant changes in tumor response induced by short-term administration of thalidomide in animals prior to treatment with the beam only would rule out a direct effect of thalidomide on tumor remission.

The finding that pretreatment with thalidomide elicited a protective effect on mucositis in dose-limiting precancerous

tissue prompted us to explore this issue further. The comparison of Groups III and IV in terms of precancerous tissue toxicity did show a slight benefit for pretreatment with thalidomide. However, since the beam-only total absorbed dose was low because Groups III and IV were set up to explore the effect of background dose and were matched for thermal neutron fluence with Groups I and II, mucositis was extremely mild. In a context of mild toxicity, the potential protective effect of thalidomide might go unnoticed. We thus set up Groups V (Th+) and VI (Th–) at a higher beam-only dose to exacerbate mucositis in precancerous tissue (without exceeding acceptable levels) and analyze the effect of thalidomide on radiation-induced mucositis, regardless of its effect on BPA delivery. The present study demonstrated a direct protective effect of thalidomide on precancerous tissue mucositis, seemingly unrelated to changes in boron compound delivery. These findings would be in keeping with the previously described protective effect of thalidomide against experimental mucositis (*47*). Given that cytokines are involved in the pathogenesis of oral mucositis (*48*), this protective effect was ascribed to the cytokine inhibitor activity of thalidomide. In particular, it was speculated that the anti-inflammatory effects could be at least partially related to tumor necrosis factor-α reduction (*47*). However, direct comparisons between this study and that of Brito *et al*. (*47*) cannot be made, because distinct differences exist between administration protocols when thalidomide is used as an antiangiogenic drug on a short-term basis only to induce a window of vascular normalization (*34* and this study) and when it is used as an anti-inflammatory drug (*47*) or as an antiangiogenic drug (*46*) on a long-term basis.

The present study provided evidence that pretreatment with thalidomide prior to BNCT mediated by BPA was therapeutically beneficial. Enhanced tumor control could be attributed to potential normalization of aberrant blood vessels and temporary restoration of adequate blood flow, enough to deliver BPA at therapeutically useful levels to otherwise inaccessible tumor areas. The fact that no statistically significant difference in tumor control was induced by pretreatment with thalidomide in animals treated with the beam only suggests that the thalidomide protocol used herein does not exert a direct effect on tumor response. The protective effect of thalidomide against precancerous tissue mucositis in animals treated with the beam only suggests that a direct effect of thalidomide could be involved in reducing toxicity.

After the withdrawal of thalidomide from the market due to its teratogenic effects (e.g. *49, 50*), it was approved in 1997 by FDA to combat a variety of dermatological conditions (*51, 52*) and to treat oral aphtous ulcers in patients with human immunodeficiency virus (HIV) infection (*53*). Within this context, potential blood vessel normalization with thalidomide prior to BPA-BNCT to optimize tumor response and reduce toxicity in the dose-limiting precancerous tissue could warrant cautious analysis in a clinical scenario. Furthermore, blood vessel normalization techniques in general also merit investigation as a way of optimizing the therapeutic advantage of BNCT in patients.

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