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Boron neutron capture therapy (BNCT) inhibits tumor development from precancerous tissue: An experimental study that supports a potential new application of BNCT

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ABSTRACT

We previously demonstrated the efficacy of boron neutron capture therapy (BNCT) mediated by boronophenylalanine (BPA), GB-10 ($Na_2^{10}B_{10}H_{10}$) and (GB-10+BPA) to control tumors, with no normal tissue radiotoxicity, in the hamster cheek pouch oral cancer model. Herein we developed a novel experimental model of field-cancerization and precancerous lesions (globally termed herein precancerous tissue) in the hamster cheek pouch to explore the long-term potential inhibitory effect of the same BNCT protocols on the development of second primary tumors from precancerous tissue. Clinically, second primary tumor recurrences occur in field-cancerized tissue, causing therapeutic failure.

We performed boron biodistribution studies followed by *in vivo* BNCT studies, with 8 months follow-up. All 3 BNCT protocols induced a statistically significant reduction in tumor development from precancerous tissue, reaching a maximum inhibition of 77–100%. The inhibitory effect of BPA-BNCT and (GB-10+BPA)-BNCT persisted at 51% at the end of follow-up (8 months), whereas for GB-10-BNCT it faded after 2 months. Likewise, beam-only elicited a significant but transient reduction in tumor development. No normal tissue radiotoxicity was observed. At 8 months post-treatment with BPA-BNCT or (GB-10+BPA)-BNCT, the precancerous pouches that did not develop tumors had regained the macroscopic and histological appearance of normal (non-cancerized) pouches.

A potential new clinical application of BNCT would lie in its capacity to inhibit local regional recurrences.

1. Introduction

We previously evidenced a remarkable therapeutic success of boron neutron capture therapy (BNCT) mediated by boronophenylalanine (BPA), GB-10 or (GB-10+BPA) to treat hamster cheek pouch tumors with no normal tissue radiotoxicity (Kreimann et al., 2001b; Trivillin et al., 2004; Trivillin et al., 2006). Despite the success of the BNCT protocols employed in these studies to treat tumors, a still unresolved challenge lies in controlling precancerous tissue. Second primary tumor locoregional recurrences that arise in field-cancerized tissue are a frequent cause of therapeutic failure (Smith and Haffty, 1999). Within this context, the hamster cheek pouch oral cancer model poses 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. Carcinogenesis protocols lead to the development of what has been termed globally herein "precancerous tissue" (field-cancerized tissue and precancerous lesions) which gives rise to the formation of tumors. Thus, this mode of tumor induction provides a tumor model surrounded by precancerous tissue, allowing for the study of the phenomenon of field-cancerization (Schwint et al., 1994; Braakhuis et al., 2003). In the present study we developed a model of precancerous tissue in the hamster cheek pouch that allows for long-term studies and mimics the phenomenon of field-cancerization in humans. Having developed the model of precancerous tissue tailored for long-term follow-up, the central aim of the present study was to evaluate the potential

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Table 1
Physical absorbed doses (Gy) for the different experimental protocols.

	Fast neutrons	Gamma-ray photons	Boron (field-cancerized tissue)	Boron (normal tissue)	Induced protons
GB-10 (50 mg boron/kg body weight) (effective irradiation time: 75 min)					
Pouch	1.04±0.09	2.90±0.10	0.12±0.01	0.12±0.01	0.30±0.03
Head	0.66±0.06	1.41±0.06	–	0.024±0.002	0.06±0.006
Body	0.33±0.03	0.53±0.02	–	0.0019±0.0002	0.01±0.001
GB-10 (34.5 mg boron/kg body weight)+BPA (31 mg boron/kg body weight) (effective irradiation time: 35 min)					
Pouch	0.48±0.04	1.34±0.05	0.054±0.005	0.054±0.005	0.14±0.01
Head	0.30±0.03	0.66±0.03	-	0.011±0.001	0.02±0.002
Body	0.16±0.01	0.24±0.01	-	0.00084±0.00008	0.002±0.0002
BPA (15.5 mg boron/kg body weight) (effective irradiation time: 55 min)					
Pouch	1.02±0.08	1.40±0.06	0.084±0.008	0.084±0.008	0.22±0.02
Head	0.72±0.06	0.95±0.04	–	0.061±0.006	0.15±0.02
Body	0.41±0.03	0.50±0.02	–	0.031±0.003	0.08±0.01

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

inhibitory effect of BNCT mediated by BPA, GB-10 or (GB-10+BPA) on the development of second primary tumors from hamster cheek pouch precancerous tissue, employing the same protocols that proved highly successful in controlling hamster cheek pouch tumors with no normal tissue radiotoxicity in previous studies (Kreimann et al., 2001b; Trivillin et al., 2006). The clinical rationale of this study was to search for a BNCT protocol that is therapeutic for tumor, not radiotoxic for the normal tissue that lies in the neutron beam path, and exerts the desired therapeutic effect on precancerous tissue, in terms of inhibition of the development of second primary tumors, without exceeding its radiotolerance.

2. Materials and methods

Model of precancerous tissue: Initial studies evaluated the adequacy of different carcinogenesis protocols in the hamster cheek pouch to yield a model of precancerous tissue amenable to long-term studies and that would guarantee tumor development in $\geqslant$80% of the animals. We treated 85 animals with the selected protocol, i.e. topical application of 0.5% DMBA in mineral oil in the right cheek pouch, twice a week for 6 weeks, and then assigned them to the different groups for boron biodistribution studies and *in vivo* BNCT studies. Studies were initiated 1 week after completion of the carcinogenesis protocol.

Institutional guidelines for the care and use of laboratory animals were followed throughout.

Boron biodistribution studies: We employed the boron compounds and the administration protocols that were proved therapeutically effective in previous tumor control studies (Kreimann et al., 2001a; Heber et al., 2004; Trivillin et al., 2006) (6 animals/group): (1) BPA (15.5 mg B/kg) ip, killed humanely at 3 h; (2) GB-10 (50 mg B/kg) iv, killed humanely at 3 h; (3) combined administration of BPA (31 mg B/kg) as fractionated ip injections; GB-10 (34.5 mg B/kg) iv, killed humanely 3 h post-administration of GB-10 and 1.5 h after the last ip injection of BPA.

Blood and tissue (precancerous pouch tissue, normal pouch tissue, liver and kidney) samples were processed for ICP-OES boron measurements.

In vivo BNCT: The hamsters were transported by plane to Bariloche, a city 1600 km south-west of Buenos Aires, to be irradiated with the thermalized epithermal beam at the RA-6 Reactor. There were a total of 67 cancerized hamsters. Thirty-three animals were divided up into 4 experimental groups, i.e. BPA-BNCT ($n = 8$), GB-10-BNCT ($n = 9$), (GB-10+BPA)-BNCT ($n = 9$) and beam-only ($n = 7$). The remaining 34 cancerized hamsters were sham-irradiated and served as controls. An additional group of 40 normal (non-cancerized) hamsters were transported to perform BNCT studies aimed at evaluating normal pouch tissue response. Thus, groups of 10 normal hamsters were treated with each of the 4 experimental protocols, i.e. BPA-BNCT, GB-10-BNCT, (GB-10+BPA)-BNCT and beam-only. In all the cases, the everted pouch and, inevitably, part of the head were placed at the beam port, which is 15 cm in diameter. The rest of the body was shielded by the lead and borated polyethylene of the beam delimiter. The average flux of thermal neutrons at the position of the pouch was $3.4 \pm 0.3 \times 10^8$ neutrons/(cm^2 s). Table 1 presents the estimated physical absorbed doses from the different radiation components for each of the experimental protocols. Table 2 shows the total physical absorbed doses and irradiation times for each experimental protocol. In the case of beam-only the hamsters were irradiated for 75 min, the longest exposure time employed for BNCT irradiations.

Table 2
Total physical absorbed doses (Gy) (mean±SD) and irradiation time.

	Beam-only	GB-10-BNCT	(GB-10+ BPA)-BNCT	BPA-BNCT
Irradiation time (min)	75	75	35	55
Precancerous tissue	4.2±0.1	7.2±1.8	4.4±1.5	4.3±1.8
Normal tissue	4.2±0.1	7.6±1.0	4.7±1.7	4.5±2.8

Follow-up: Precancerous tissue response and potential tumor development from precancerous tissue were assessed weekly by visual inspection and tumor volume assay (when pertinent) for 8 months after treatment with the 4 different experimental protocols. Likewise, normal pouch tissue response was assessed in the normal (non-cancerized) hamsters treated with each of the 4 experimental protocols. Controls (cancerized hamsters submitted to sham-irradiation) were followed in the same way. The clinical signs and body weight of the animals were monitored regularly. At different time-points, 1–2 animals per protocol that had already developed second primary tumors were killed humanely for histological analysis of tumor, precancerous pouch tissue and normal pouch tissue.

The quantitative end-points that were evaluated were the accumulated percentage of animals that developed second primary tumors from precancerous tissue, % inhibition induced by the different treatment protocols referred to tumor development from

sham-irradiated precancerous tissue, volume of tumors that did eventually develop from precancerous tissue and T50 (the time to appearance of tumors in 50% of the hamsters). The few animals that had already developed tumor that were killed humanely for histological analysis were inevitably lost to follow-up. However, because they had already developed at least one tumor when they were killed humanely, they were included, until the last time-point, in the accumulated percentage of animals that developed second primary tumors.

When pertinent, statistical analysis of the data was performed employing the repeated measures ANOVA experimental design. At selected representative time-points, the different treatment groups and the control group were compared employing a Chi-square test with Yates' correction. Statistical significance was set at $p \leqslant 0.05$.

3. Results

3.1. Boron biodistribution studies

Table 3 presents the most relevant boron content absolute values and ratios for each of the boron compound administration protocols. In all the cases, absolute boron uptake in precancerous tissue fell within a therapeutically useful range. The boron content ratio precancerous tissue/normal pouch tissue revealed no preferential uptake by precancerous tissue.

3.2. In vivo BNCT

Evaluation of radiotoxicity: None of the cancerized or normal (non-cancerized) animals treated with any of the experimental protocols exhibited clinical signs of radiotoxicity throughout the follow-up period. In some cases, the precancerous pouch tissue treated with the BNCT protocols exhibited early, slight and reversible mucositis that resolved by the third week post-treatment. This effect was somewhat greater and more frequent in the BPA-BNCT group. The normal pouches treated with the experimental protocols were indistinguishable from untreated normal pouches on visual inspection and on histological analysis until the last time-point evaluated.

Tumor development from precancerous tissue: Fig. 1 shows the accumulated percentage of animals that developed second primary tumors from precancerous tissue at representative time-points for each of the treatment protocols and controls (cancerized, sham-irradiated animals). The development of second primary tumors from precancerous tissue in controls represents the kinetics of tumor development in the model of precancerous tissue developed herein. At 2 weeks post-treatment the beam-only, BPA-BNCT and (GB-10+BPA)-BNCT protocols exerted a reduction in tumor development vs. controls. However, this difference did not reach statistical significance. At 1 month and 2 months post-treatment all 4 treatment groups exhibited significantly lower values than controls ($p = 0.001$ and 0.03, respectively). At 4, 6 and 8 months post-treatment, the values corresponding to BPA-BNCT and (GB-10+BPA)-BNCT were similar in both groups and significantly lower than controls ($p < 0.05$, $p = 0.015$, $p = 0.002$, respectively). At these time-points, the GB-10-BNCT and beam-only groups no longer differed from controls ($p > 0.05$).

Based on these data, we calculated the percentage inhibitory effect of the different treatment protocols on the development of second primary tumors from precancerous tissue at representative time-points, referred to tumor development in the control group (Table 4). All 3 BNCT protocols induced an inhibitory effect on tumor development from precancerous tissue, reaching a maximum 77–100% inhibition. The inhibitory effect of BPA-BNCT and (GB-10+BPA)-BNCT persisted at 51% at the last time-point evaluated (8 months). The inhibitory effect of GB-10-BNCT disappeared after 2 months. Similarly to GB-10-BNCT, beam-only exerted a transient inhibitory effect that faded after 2 months.

The values of T50 (the time to appearance of tumors in 50% of the hamsters) further supported the inhibitory effect observed for all 4 experimental protocols, in particular for BPA-BNCT and (GB-10+BPA)-BNCT, i.e. control: 4–5 weeks; GB-10-BNCT: 9–10 weeks; beam-only: 11 weeks; BPA-BNCT: not reached within the follow-up period of 32 weeks; (GB-10+BPA)-BNCT: not reached within the follow-up period of 32 weeks.

Up to 5 weeks post-sham-irradiation, the cancerized, sham-irradiated animals (controls) developed tumors smaller than 10 mm^3. As from 5 weeks these control animals began to develop

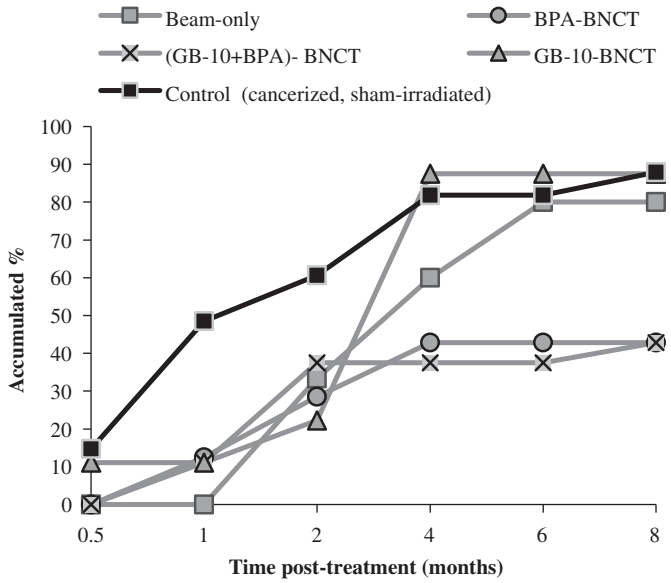

Fig. 1. Accumulated % of animals that developed second primary tumors from precancerous tissue at representative time-points for the different experimental protocols.

Table 3
Boron concentration (ppm) (mean ± SD).

	GB-10	GB-10 +BPA	BPA
Precancerous tissue	24.4 ± 9.7 ($n = 23$)	45.1 ± 15.3 ($n = 24$)	19.7 ± 9.4 ($n = 23$)
Normal tissue	27.6 ± 17.8 ($n = 6$)	49.8 ± 20.4 ($n = 6$)	21.9 ± 10.9 ($n = 6$)
Blood	18.1 ± 7.3 ($n = 6$)	24.2 ± 11.1 ($n = 8$)	4.8 ± 2.4 ($n = 6$)
Precancerous tissue/ normal tissue	0.9/1	0.9/1	0.9/1

Table 4
Percentage inhibitory effect of the different experimental protocols on the development of second primary tumors from precancerous tissue at representative time-points, referred to tumor development in the control group (cancerized, sham-irradiated).

Time post-treatment (months)	Beam-only (%)	GB-10-BNCT (%)	(GB-10+BPA)-BNCT (%)	BPA-BNCT (%)
0.5	100	26	100	100
1	100	77	77	73
2	46	64	38	52
4	27	0	54	48
6	2	0	54	48
8	9	0	51	51

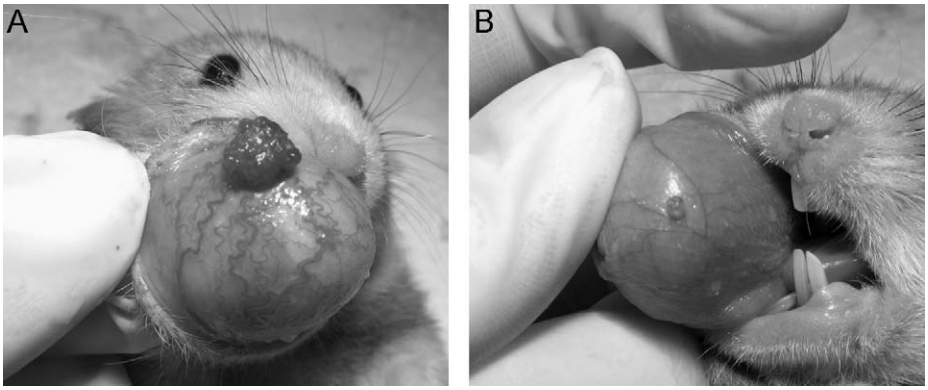

Fig. 2. Representative example of untreated pouch and treated pouch that developed tumor: (A) control (cancerized, sham-irradiated) cheek pouch (arrow: tumor, volume 132 mm^3); (B) cancerized cheek pouch treated with (GB-10+BPA)-BNCT (arrow: tumor, volume 2.25 mm^3). Note the difference in tumor size between both groups.

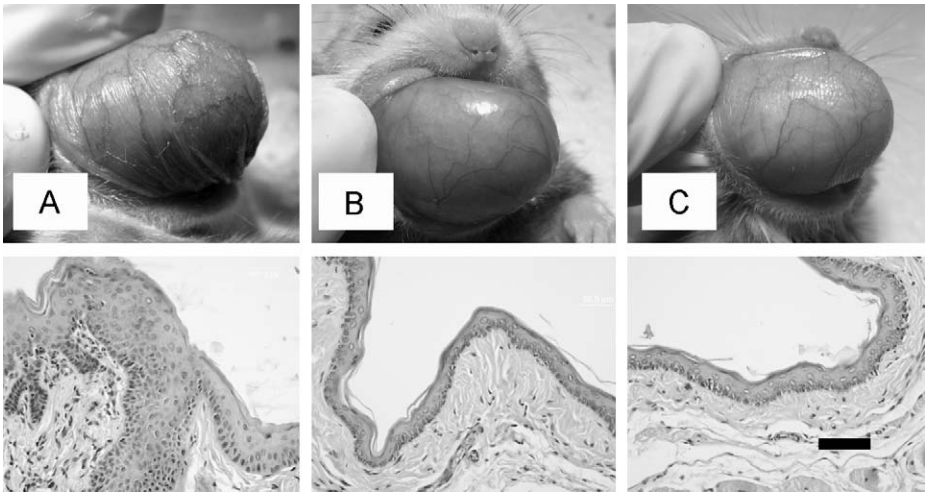

Fig. 3. (A) Control (not treated) field-cancerized pouch; (B) field-cancerized pouch 7 months post-BPA-BNCT; (C) normal pouch (non-cancerized, not treated). Below, in each case, we show the corresponding characteristic light microscopy images (40 ×, H&E). Bar: 70 μm.

tumors in the 10–50 mm^3 volume range and even larger than 50 mm^3. Conversely, as from 5 weeks post-treatment and until the last time-point evaluated (8 months), the tumors of the few animals that did exhibit tumor development in the BPA-BNCT and (GB-10+BPA)-BNCT groups never exceeded volumes of 10 mm^3 (Fig. 2).

At 8 months post-treatment with BPA-BNCT or (GB-10+BPA)-BNCT, the precancerous pouches that did not develop tumors had regained the macroscopic and histological appearance of normal (non-cancerized) pouches (Fig. 3).

4. Discussion and conclusions

All the BNCT protocols employed herein exerted a marked, statistically significant effect on the development of second primary tumors from precancerous tissue with no normal tissue radiotoxicity. The inhibitory effect of GB-10-BNCT lasted for approximately 2 months, whereas in the case of BPA-BNCT and (GB-10+BPA)-BNCT a 51% inhibition persisted at the last time-point evaluated (8 months post-treatment).

The differential effect of BNCT on precancerous tissue and normal tissue cannot be attributed to preferential boron uptake by precancerous tissue and might be due to one or more of the following effects: differences in CBE values, preferential microlocalization of BPA in precancerous foci at a higher risk of malignant transformation and vascular targeting of GB-10-BNCT that would impair the process of angiogenesis associated to tumor microenvironment development. The effect of GB-10-BNCT would fade similarly to the effect of beam-only. The overall more conserved structure and function of precancerous tissue blood vessels compared to tumor blood vessels would reduce the CBE value for GB-10 in precancerous tissue, compared to its known efficacy in tumor (Trivillin et al., 2006).

Overall, the inhibitory effect on precancerous tissue could be due to the cellular and/or vascular targeting of foci of precancerous change more liable to malignant transformation (Heber et al., 2007), to the effect on the tissue microenvironment (Laconi et al., 2008) or both.

The present study provides, for the first time, evidence that BNCT induces a long-term marked inhibitory effect on tumor development from precancerous tissue, with no normal tissue radiotoxicity and without exceeding precancerous tissue radiotolerance. Furthermore, we showed that BNCT is capable of reverting at least the histological hallmarks of premalignancy. Thus, the BNCT protocols that were previously proved effective to control established tumors would also inhibit locoregional recurrences caused by the development of tumors in precancerous tissue, suggesting a novel potential application of BNCT.

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