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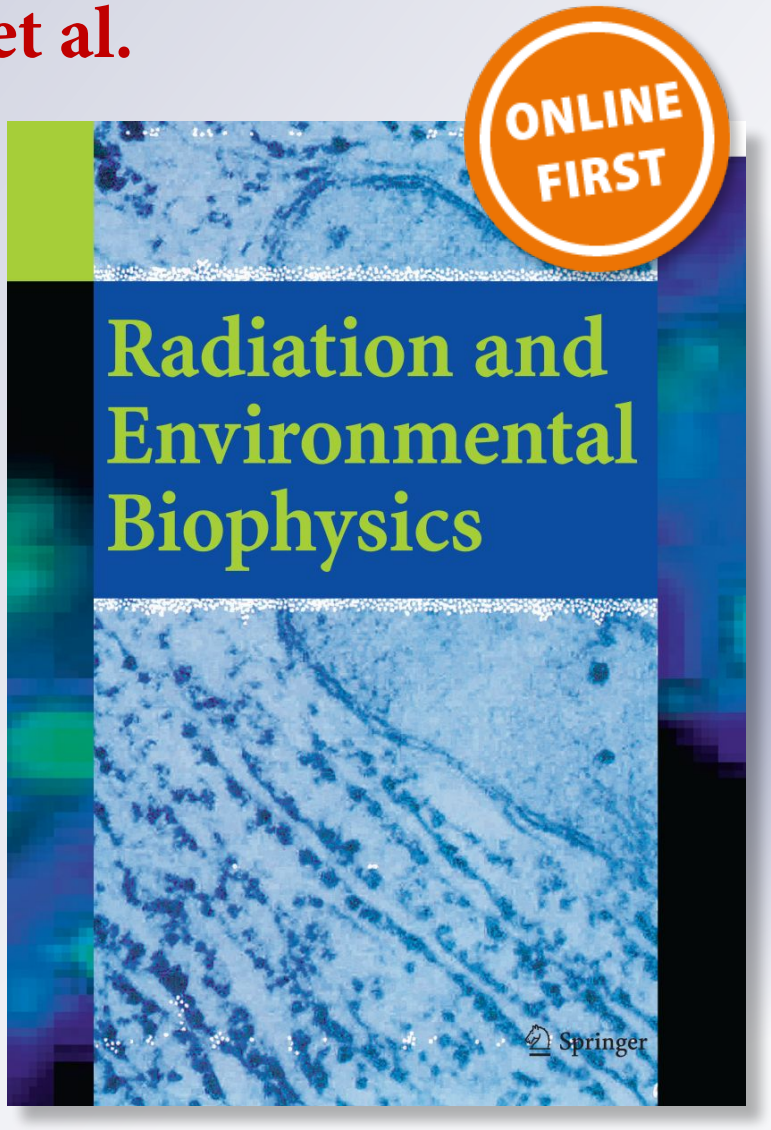


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ORIGINAL ARTICLE

# Abscopal effect of boron neutron capture therapy (BNCT): proof of principle in an experimental model of colon cancer

**Verónica A. Trivillin[1,2] · Emiliano C. C. Pozzi[3] · Lucas L. Colombo[2,4] · Silvia I. Thorp[5] · Marcela A. Garabalino[1] · Andrea Monti Hughes[1,2] · Sara J. González[2,5] · Rubén O. Farías[5] · Paula Curotto[3] · Gustavo A. Santa Cruz[6] · Daniel G. Carando[2,7] · Amanda E. Schwint[1,2]**

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**Abstract** The aim of the present study was to evaluate, for the first time, the abscopal effect of boron neutron capture therapy (BNCT). Twenty-six BDIX rats were inoculated subcutaneously with $1 \times 10^6$ DHD/K12/TRb syngeneic colon cancer cells in the right hind flank. Three weeks post-inoculation, the right leg of 12 rats bearing the tumor nodule was treated with BPA-BNCT (BPA-Boronophenylalanine) at the RA-3 nuclear reactor located in Buenos Aires, Argentina, at an absorbed dose of 7.5 Gy to skin as the dose-limiting tissue. The remaining group of 14 tumor-bearing rats were left untreated and used as control. Two weeks post-BNCT, $1 \times 10^6$ DHD/K12/TRb cells were injected subcutaneously in the contralateral left hind flank of each of the 26 BDIX rats. Tumor volume in both legs was measured weekly for 7 weeks to determine response to BNCT in the right leg and to assess a potential influence of BNCT in the right leg on tumor development in the left leg. Within the BNCT group, a statistically significant reduction was observed in contralateral left tumor volume in animals whose right leg tumor responded to BNCT (post-treatment/pre-treatment tumor volume <1) versus animals who failed to respond (post/pre ≥1), i.e., 13 ± 15 vs 271 ± 128 $mm^3$. In addition, a statistically significant reduction in contralateral left leg tumor volume was observed in BNCT-responsive animals (post/pre <1) vs untreated animals, i.e., 13 ± 15 vs 254 ± 251 $mm^3$. The present study performed in a simple animal model provides proof of principle that the positive response of a tumor to BNCT is capable of inducing an abscopal effect.

**Keywords** Boron neutron capture therapy · BNCT · Abscopal effect · Boronophenylalanine (BPA) · Nuclear reactor RA-3

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## Introduction

The abscopal (*ab-scopus*, away from the target) effect, originally described by Mole (1953), refers to the inhibitory action of radiotherapy on the development and growth of non-targeted tumors, i.e., at a site distant from the area of irradiation (e.g., Okuma et al. 2011; Golden et al. 2013). This phenomenon has been reported to occur in the treatment of a variety of malignancies including hepatocellular carcinoma, lymphoma, papillary adenocarcinoma, adenocarcinoma of the esophagus, chronic lymphocytic leukemia, and melanoma (e.g., Sham 1995; Camphausen et al. 2003; Grimaldi et al. 2014). Local standard radiotherapy on cancer

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cells has occasionally been shown to induce regression of metastatic cancer at distant sites which have not been irradiated (Park et al. 2014).

Preclinical evidence suggests that the abscopal effect may be mediated by radiation-induced immune responses (Demaria et al. 2004). Tumors become clinically apparent once they have escaped from immune destruction by creating an immunosuppressive microenvironment. Radiotherapy could cause immunogenic cell death resulting in cross-priming of tumor-specific T cells, conceivably acting as an in situ tumor vaccine (Vatner et al. 2014). Similarly, the administration of attenuated or genetically modified microorganisms has been shown to stimulate the immune system, inducing tumor regression (Kucerova and Cervinkova 2016). Radiotherapy would induce and enhance the endogenous anti-tumor innate and adaptive tumor response (Park et al. 2014). Although the mechanisms involved in the abscopal effect are still unclear (Okuma et al. 2011), they would include movement of lymphocytes into the tumor microenvironment, upregulation of tumor antigens and antigen-presenting machinery, and induction of positive immunomodulatory pathways (Park et al. 2014; Lock et al. 2015). Although irradiation of lymphoid sites affected by disease will be more effective in triggering systemic immunity because immune effectors trafficking through these regions are more likely to encounter released antigen, irradiation of visceral sites of disease including bone, skin, and parenchyma are also capable of inducing an abscopal effect (e.g., Okuma et al. 2011; Park et al. 2014; Takaya et al. 2007). Overall, reports of abscopal responses in clinical practice have been infrequent, suggesting that radiotherapy alone may be insufficient to induce a sustained systemic antitumor effect. However, coupling irradiation with immunotherapy could amplify the radiation-induced immune response sufficiently to elicit a robust abscopal effect (Golden et al. 2013; Demaria et al. 2005; Hodge et al. 2012).

To date, all the experimental, preclinical, and clinical data on the abscopal effect refer to standard radiotherapy. Therefore, it is compelling to evaluate the potential abscopal effect of boron neutron capture therapy (BNCT). In BNCT, $^{10}$B-carriers that are taken up preferentially by tumor are administered followed by irradiation with thermal or epithermal neutrons. Due to the neutron capture reaction $^{10}B(n,\alpha)^{7}Li$, high linear energy transfer (LET) alpha particles (1.47 MeV) and recoiling $^{7}$Li nuclei (0.84 MeV) are produced. These high-LET particles (range in tissue: 5–9 μm) have a high relative biological effectiveness, causing damage mainly in the cell where the capture reaction occurs (Coderre and Morris 1999). If the boron compound targets tumor tissue with some degree of selectivity, BNCT would damage tumor preferentially, with less damage to healthy tissue in the target volume. In addition to the tumor specific boron dose component from alpha and $^{7}$Li particles, (1) low-LET gamma rays in the beam, (2) low-LET gamma rays resulting from the capture reaction $^{1}H(n_{th},\gamma)^{2}H$, (3) high-LET protons produced by the scattering of fast neutrons, and (4) high-LET protons resulting from the capture reaction $^{14}N(n_{th},p)^{14}C$ constitute the non-specific background dose and also contribute to absorbed dose. Relative biological effectiveness (RBE) and compound biological effectiveness (CBE) factors of the involved radiation qualities will govern the biologically effective dose (Coderre and Morris 1999), which should be calculated as a photon-isoeffective dose as recently established (González and Santa Cruz 2012). BNCT protocols maximize the tumor-specific boron radiation dose and minimize the background dose that affects tumor and healthy tissues similarly (e.g., Coderre and Morris 1999; Kreimann et al. 2001a, b; Trivillin et al. 2006; Molinari et al. 2011, 2012). Clinical trials of BNCT for different tumors, i.e., glioblastoma multiforme, melanoma, head and neck tumors, and liver metastases (González et al. 2004; Kankaanranta et al. 2012; Miyatake et al. 2014; Wang et al. 2014; Yanagie et al. 2014), have shown a potential therapeutic advantage, with room for improvement. Translational studies in adequate experimental models have contributed to the knowledge of BNCT radiobiology, allowing for the optimization of this technique for different tumor types and localizations (e.g., Barth 2015). The authors' group has comprehensively studied the radiobiology of BNCT in different experimental models to optimize BNCT for the treatment of different pathologies (e.g., Kreimann et al. 2001a, b; Trivillin et al. 2006; Molinari et al. 2011, 2012; Garabalino et al. 2013; Monti Hughes et al. 2013, 2015; Pozzi et al. 2012, 2013; Heber et al. 2014; Schwint and Trivillin 2015) and has performed preclinical studies with encouraging results (Rao et al. 2004; Trivillin et al. 2008).

Standard photon radiotherapy uses low-LET radiation, fractionated protocols, multiple beams irradiating the target volume from various directions, and multileaf collimators. Because of the duration of the treatment periods (5–6 weeks), anatomical changes and uncertainties in patient positioning require individualized treatment planning adjusted over time. BNCT is based mainly on the effect of high-LET radiation with a high relative biological effectiveness. Generally, it involves only single or, at most, double applications, which increases the patients' quality of life and reduces the impact of anatomical changes on dose delivery to the tumor. Because tumor targeting relies on the preferential incorporation of boron compounds to tumor cells, BNCT might be particularly useful to treat micrometastasis, infiltrating malignant cells, and foci of malignant transformation. Furthermore, the conformation of 3D dosimetry is improved and variations due to positioning are reduced. Finally, BNCT can be employed for the treatment of recurrent lesions that have already been exposed to photon radiotherapy (Kankaanranta et al. 2012; Hopewell et al. 2011;

Khan and Gerbi 2012). As a disadvantage, BNCT is not suited to cure systemic disease (Monti Hughes et al. 2015). However, if BNCT were capable of eliciting an abscopal effect, treatment of a primary tumor might contribute to the control of disseminated disease.

Given the differences between standard photon radiotherapy (for which the abscopal effect has been described) and BNCT, the aim of this study was to assess, for the first time, the potential abscopal effect of BNCT using a simple experimental model. Admittedly, the relatively weak systemic abscopal effect induced by standard radiotherapy alone could be enhanced by adding immune manipulation. However, there is evidence that radiation alone is sufficient to provide the necessary signals for cross-priming of cytotoxic T-lymphocytes against tumor antigens (Vatner et al. 2014). In the present study the abscopal effect of BNCT was investigated, without the potential boosting effect of immunotherapy.

## Materials and methods

### Experimental model

A total of 26 male or female adult BDIX rats (Charles River Lab., MA, USA), 170–250 g body mass (bm), were used in this study. The animals were housed as previously described (e.g., Pozzi et al. 2013). All rats were injected subcutaneously in the right hind flank, under ketamine (36.5 mg/kg bm)–xylazine (5.4 mg/kg bm) anesthesia, with $1 \times 10^6$ DHD/K12/TRb syngeneic colon cancer cells (ECACC, UK) in 100 µl of F10-DMEM culture medium (GIBCO) using a syringe with a 27-gauge needle. The inoculation protocol was selected based on a pilot study in which tumor growth and histology and clinical status of the animals were monitored in different groups of animals for different inoculation protocols and at different times post-inoculation. On the basis of these findings, all subsequent experiments were performed 3 weeks post-inoculation, when the animals developed subcutaneous measurable, vascularized tumor nodules. This time point was considered as T0.

### Biodistribution studies

Biodistribution studies were performed to estimate boron concentration in tumor, blood, and clinically relevant normal tissues. These boron concentration values were used to calculate the boron component of the dose for the different tissues/organs and, in turn, perform dose calculations for BNCT. BPA biodistribution studies were performed in a group of three rats bearing one tumor nodule each, induced as described above. BPA (L-enantiomer, 98% enriched in $^{10}$B, Interpharma, Praha a.s.) was prepared as previously described (Garabalino et al. 2011) as 0.42 M BPA-fructose in United States Pharmacopeia (USP) water for injection and administered intravenously at a dose of 46.5 mg $^{10}$B/kg bm in the jugular vein, surgically exposed under ketamine (36.5 mg/kg bm)–xylazine (5.4 mg/kg bm) anesthesia. Samples of blood, tumor, and skin were taken 3 h post-administration of BPA and processed as previously described (Garabalino et al. 2011) for gross boron measurement by inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC2, Perkin Elmer).

### In vivo BNCT

Three weeks post-inoculation of DHD/K12/TRb colon carcinoma tumor cells, 12 tumor-bearing rats were treated with BNCT. Irradiations were performed at a thermal neutron source constructed for biomedical applications by the National Atomic Energy Commission of Argentina at the RA-3 research and production nuclear reactor facility located in Buenos Aires (Miller et al. 2009; Pozzi et al. 2013). Through a tunnel penetrating the graphite structure of the thermal neutron column, the animals were inserted into a near-isotropic neutron field while the reactor was in normal operation (Fig. 1). Neutron flux measurements were performed with a self-powered neutron detector (SPND) (Miller et al. 2004) at a monitor position during each irradiation, to check for potential neutron flux variations and estimate the exposure time required to reach the prescribed dose. BPA was administered intravenously at a dose of 46.5 mg $^{10}$B/kg bm in the jugular vein, as described above. Dosimetric calculations were based on previously reported physical dosimetry data for the RA-3 facility (Pozzi et al. 2009) and BPA boron biodistribution data reported herein for this model.

The right leg bearing the tumor nodule was locally irradiated 3 h post-administration of BPA. A lithium carbonate thermal neutron shield (enriched to 95% in lithium-6), fabricated ad hoc, was used to protect the body of the animal from the thermal neutron flux while exposing the leg through a collimated aperture (Fig. 1).

An absorbed dose of 7.5 Gy was administered to exposed skin. Dose was prescribed not to exceed the maximum tolerated dose of 18 Gy-eq to skin as the dose-limiting tissue (González et al. 2009). In BNCT, dose prescription is based on the well-known tolerance dose for photon irradiation corresponding to the "organ at risk" within the treatment volume. While the tolerance dose values for photon irradiation are based on a large number of studies and are considered robust, fewer data are available in the literature on the tolerance dose for BNCT. The dose components with different LET characteristics in BNCT will have varying degrees of biological effectiveness with regard to tumor and to the various normal tissues within the treatment volume. Thus, to express the total BNCT

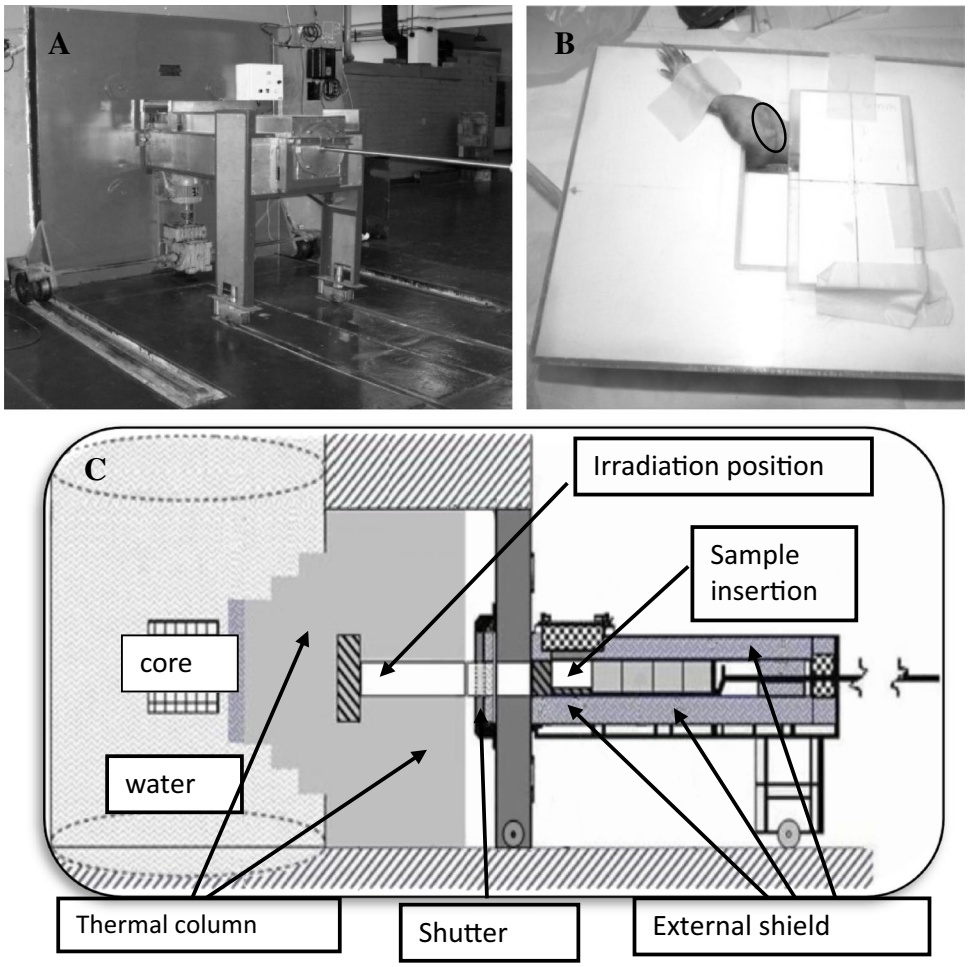


**Fig. 1** **a** RA-3 thermal facility; **b** the right leg bearing the tumor nodule was locally irradiated employing a lithium carbonate thermal neutron shield (enriched to 95% in lithium-6), fabricated ad hoc, to protect the body of the animal from the thermal neutron flux while exposing the leg through a collimated aperture. The tumor area is encircled in *black*; **c** diagram of RA-3 thermal facility showing the tunnel penetrating the graphite structure of the thermal neutron column and the shutter and shields that enable the insertion of samples into a near-isotropic neutron field while the reactor is in normal operation

dose in a common, photon-equivalent unit (Gy-eq), enabling comparison with conventional photon irradiation, biological effectiveness must be considered in the calculations (Coderre and Morris 1999; González and Santa Cruz 2012).

Table 1 presents the prescribed absorbed doses from the different radiation components, the total BNCT dose, and the gross boron concentration data used for dose calculations. Irradiation time to reach the prescribed dose was 13 min. The thermal neutron flux at the irradiation position was $3.6 \times 10^9$ n cm$^{-2}$ s$^{-1}$, while the thermal neutron flux at all the locations within the shield container was at least a factor of 20 lower than the flux on the exposed leg. Mean gamma dose rate at the irradiation position was estimated as 4.9 Gy h$^{-1}$.

The remaining group of 14 time-matched tumor-bearing rats were not treated with BNCT and used as control. At 2 weeks post-BNCT, $1 \times 10^6$ DHD/K12/TRb cells were injected subcutaneously as previously described in the contralateral left hind flank of each of the 26 BDIX rats. This time interval was chosen based on experimental studies of abscopal effect induced by standard radiotherapy by other authors (Zenkoh et al. 2015). Admittedly, multiple protocols that model different clinical scenarios would warrant testing. In addition, in the present experimental set-up, the tumor in the contralateral left leg should not be exposed to any

**Table 1** Absorbed doses (Gy) for the different tissues

| Tissue | ppm $^{10}$B mean ± SD | Total γ ray dose | Induced protons (N-14) | Boron dose | Total BNCT dose |
|---|---|---|---|---|---|
| Tumor | 20.1 ± 4.5 | 1.1 [0.9; 1.3] | 1.0 [0.9; 1.0] | 6.0 [5.6; 6.3] | 8.1 [7.3; 8.7] |
| Leg (skin) | 18.2 ± 2.4 | 1.1 [0.9; 1.3] | 1.0 [0.9; 1.0] | 5.5 [5.0; 5.7] | 7.5 [6.8; 8.1] |

BNCT dose components, total BNCT dose, and the gross boron concentration values for tumor and leg skin employed for dose calculations. The data are presented as Mean [min; max]

level of irradiation dose to avoid a potential contribution of this dose to tumor growth inhibition. This is a concern in protocols where the exposed tumor and the "out of field" tumor are both present in the animal at the time of treatment (Camphausen et al. 2003). In the present experiment, the lithium carbonate shield (enriched to 95% in lithium-6) used for BNCT irradiations protects the body of the animal from the thermal neutron flux but does not confer protection against the gamma radiation dose that is part of the background dose in BNCT as described above. Thus, to avoid a potential influence of gamma dose on tumor growth in the left leg a two-stage inoculation protocol was used here.

### Follow-up

Tumor volume in the right leg was determined pre-BNCT and once a week post-BNCT for 7 weeks in BNCT-treated and untreated animals by external caliper measurement of the three largest orthogonal diameters ($d_i$) and calculated as d1 × d2 × d3 as previously described (e.g., Molinari et al. 2012). Likewise, tumor volume was measured weekly in the contralateral left flank to assess a potential influence of the response of BNCT-treated tumors in the right leg on tumor development in the contralateral left leg. This potential inhibitory effect on tumor development in the left leg, induced by a positive response to BNCT of the tumor in the right leg, was used as an indicator of abscopal effect of BNCT. Clinical signs and local toxicity were monitored throughout.

Animal experiments were carried out in accordance with the guidelines laid down by the National Institute of Health in the USA regarding the care and use of animals for experimental procedures and in accordance with protocols approved by the National Atomic Energy Commission Animal Care and Use Committee (CICUAL-CNEA).

### Statistical analysis

The statistical significance of the differences in tumor volume was evaluated by an unpaired, two-tailed Student´s t test. Statistical significance was set at $p = 0.05$.

## Results

Gross boron concentration was 20.1 ± 4.5 ppm for tumor, 18.2 ± 2.4 ppm for skin, and 13.2 ± 0.5 for blood. Boron concentration in tumor fell within a therapeutically useful range (e.g., Trivillin et al. 2006; Garabalino et al. 2011). These boron concentration values were used to calculate the boron component of the BNCT dose.

No clinical systemic signs of toxicity were observed in the BNCT and non-treated groups of tumor-bearing rats. The only sign of local toxicity was moist desquamation in the exposed skin of the BNCT group. Mean tumor volume in the right leg of BNCT-treated animals was 104 ± 114 $mm^3$ ($n = 12$) at 7 weeks post-BNCT. In time-matched untreated animals, tumor volume in the right leg was significantly larger ($p < 0.0001$), i.e., 2284 ± 1552 $mm^3$ ($n = 14$). Tumor response to BNCT was considered positive when post-treatment tumor volume was smaller than tumor volume at the time of irradiation (post-treatment/pre-treatment tumor volume ratio <1). Employing this criterion, of the 12 BNCT-treated tumors (right leg), 5 were considered responsive ($BNCT_R$). The mean tumor volume of these five responsive tumors at 7 weeks post-treatment was 16 ± 23 $mm^3$, whereas the mean tumor volume of the seven remaining non-responsive tumors (post/pre tumor volume ratio ≥1) in the right leg treated with BNCT was 167 ± 110 $mm^3$. Although tumors were considered "non-responsive" (BNCT*) when post/pre tumor volume ratio ≥1, the mean tumor volume in this "non-responsive" group was significantly ($p = 0.0021$) smaller than for non-treated tumors (167 ± 110 vs 2284 ± 1552 $mm^3$). The difference in tumor volume between responsive and non-responsive right leg tumors treated with BNCT was statistically significant ($p = 0.0137$). In all cases, the spread in tumor volume values was large.

Tumor growth in the contralateral left leg was used as an end point to evaluate a potential abscopal effect of right leg tumor response to BNCT on left leg tumor development. Tumor volume in the contralateral left leg was smaller (albeit not significantly) in animals treated with BNCT in the right leg than in untreated animals (164 ± 163 $mm^3$, $n = 12$ vs. 254 ± 251 $mm^3$, $n = 14$, respectively). Within the BNCT group, a statistically significant reduction was observed in left leg tumor volume in animals whose right leg tumor responded to BNCT (post-treatment/pre-treatment tumor volume ratio <1) vs animals who failed to respond to BNCT (post/pre ≥1), i.e., 13 ± 15 $mm^3$, $n = 5$ vs. 271 ± 128 $mm^3$, $n = 7$ ($p = 0.0013$). In addition, a statistically significant reduction in left leg tumor volume was observed in BNCT-responsive animals versus untreated animals, i.e., 13 ± 15 $mm^3$, $n = 5$ vs 254 ± 251 $mm^3$, $n = 14$, respectively ($p = 0.05$). Table 2 shows individual data for each of the 26 rats. It is interesting to note that all the rats that were considered responsive to BNCT (using the criterion post/pre tumor volume ratio <1) and exhibited an abscopal effect, were female. However, not all female rats responded to BNCT (using the post/pre tumor volume ratio <1 criterion) and exhibited an abscopal effect. Figure 2 shows the response of the right leg tumor for the different groups (top panel) at 7 weeks post-treatment and the correlation with tumor volume in the left leg (lower panel). An abscopal effect is observed in the tumor of the contralateral (left) leg of the group of

**Table 2** Individual data for each of the rats

| Characteristic | | | T0 | | 7 weeks post-T0 | | | |
|---|---|---|---|---|---|---|---|---|
| Rat | Gender | Group | Body mass (g) | Right leg TV ($mm^3$) | Body mass (g) | Right leg TV ($mm^3$) | Post/pre TV ratio | Left leg TV ($mm^3$)[#] |
| R13 | F | $BNCT_R$ | 203.5 | 43 | 207.9 | 0 | 0.00 | 8 |
| R16 | F | $BNCT_R$ | 194.9 | 53 | 192.9 | 26 | 0.50 | 39 |
| R32 | F | $BNCT_R$ | 191.1 | 67 | 181.4 | 0 | 0.00 | 13 |
| R33 | F | $BNCT_R$ | 204.8 | 54 | 188.7 | 52 | 0.97 | 7 |
| R36 | F | $BNCT_R$ | 195.4 | 43 | 197.1 | 0 | 0.00 | 0 |
| R11 | F | BNCT* | 194.0 | 46 | 184.5 | 65 | 1.43 | 61 |
| R18 | M | BNCT* | 211.0 | 24 | 212.9 | 28 | 1.20 | 243 |
| R22 | M | BNCT* | 215.7 | 88 | 259.5 | 153 | 1.73 | 331 |
| R28 | M | BNCT* | 247.7 | 179 | 326.4 | 355 | 1.99 | 293 |
| R31 | F | BNCT* | 188.6 | 122 | 171.2 | 184 | 1.51 | 237 |
| R39 | M | BNCT* | 274.7 | 134 | 302.5 | 248 | 1.85 | 245 |
| R41 | M | BNCT* | 258.4 | 118 | 279.2 | 136 | 1.15 | 488 |
| R12 | F | C | 229.1 | 61 | 217.8 | 1036 | 16.99 | 126 |
| R14 | F | C | 212.5 | 57 | 213.8 | 809 | 14.14 | 118 |
| R15 | F | C | 199.1 | 44 | 185.9 | 958 | 21.83 | 279 |
| R17 | F | C | 195.0 | 63 | 180.4 | 635 | 10.06 | 105 |
| R19 | M | C | 261.1 | 78 | 301.2 | 1894 | 24.16 | 125 |
| R20 | M | C | 201.7 | 70 | 284.8 | 2086 | 29.78 | 189 |
| R27 | M | C | 229.5 | 144 | 307.7 | 3430 | 23.82 | 379 |
| R29 | M | C | 221.9 | 137 | 315.7 | 5652 | 41.17 | 1023 |
| R30 | F | C | 162.7 | 74 | 164.3 | 1950 | 26.35 | 244 |
| R34 | F | C | 192.1 | 70 | 191.4 | 1415 | 20.09 | 128 |
| R35 | F | C | 181.8 | 57 | 193.0 | 2856 | 49.67 | 116 |
| R37 | F | C | 193.0 | 65 | 190.2 | 1146 | 17.76 | 189 |
| R38 | M | C | 287.5 | 164 | 328.7 | 3189 | 19.44 | 50 |
| R40 | M | C | 280.4 | 265 | 343.8 | 4923 | 18.61 | 485 |

*T0* Three weeks post-inoculation of tumor cells in the right leg (pre-BNCT), $BNCT_R$ responsive to BNCT (post/pre TV ratio <1), *BNCT** post/pre TV ratio ≥1, *C* control (untreated), *TV* tumor volume

[#] Five weeks post-inoculation of tumor cells in the left contralateral leg. Data corresponding to each of the rats at T0 (pre-treatment) and at the last time point evaluated (7 weeks post-treatment). The TV values of the exposed right leg pre and post-treatment are shown and used to determine the post/pre TV ratio and establish if the tumor was responsive. The TV values of the contralateral (left) leg were low (0–39 $mm^3$) when the tumor in the right leg was responsive

animals whose right leg tumor responded to BNCT (using the criterion post/pre tumor volume <1). Complementarily, Fig. 3 shows the volume of the right leg and contralateral left leg tumors as a function of time post-treatment of the right leg tumor, evidencing the response of the right leg tumor to BNCT over time and the associated abscopal response over time in the BNCT-R group. Figure 4 shows a representative example of abscopal effect, i.e., a responsive tumor in the right leg treated with BNCT alongside a small tumor in the left contralateral leg as compared to an untreated tumor in the right leg alongside a large tumor in the contralateral left leg.

A consistent and robust abscopal effect of BNCT was identified as an inhibitory effect on tumor growth in the contralateral left (untreated) leg elicited by a positive local response to BNCT of the tumor in the right leg.

## Discussion

To date, the abscopal effect has been described in association with localized standard radiotherapy (e.g., Postow et al. 2012). Local irradiation would induce a release of mitotic inhibitors (cytokines such as tumor necrosis factor α) into the circulation that mediate a systemic antitumor effect (Camphausen et al. 2003; Ohba et al. 1998). Furthermore, irradiation of tumor in one site would induce the release of the circulating tumor antigen or inflammatory factors that

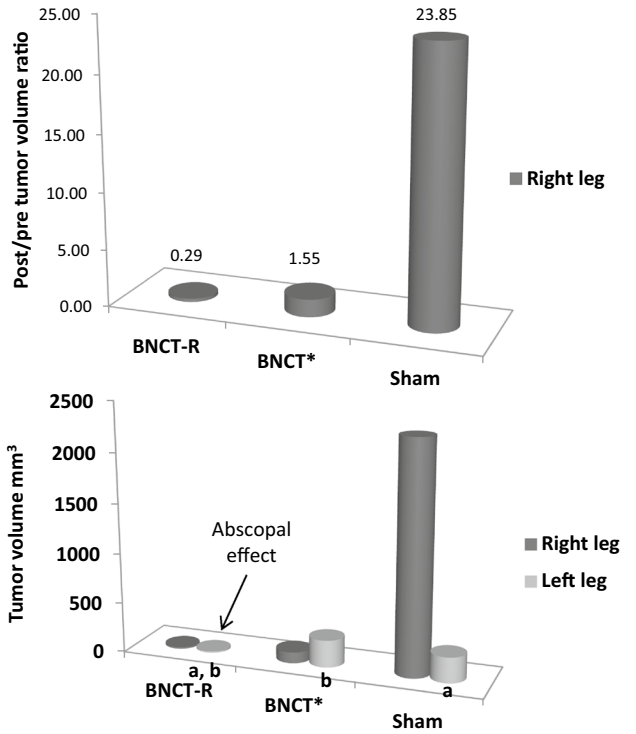


**Fig. 2** *Top panel* right leg tumor response at 7 weeks post-treatment in terms of mean post/pre tumor volume ratio for the group of BNCT responders (<1) (BNCT-R); the BNCT group considered to be non-responders (≥1) (although tumor volume was significantly lower than for the Sham group) (BNCT*), and the control Sham group. *Lower panel* tumor volume 7 weeks post-treatment for the right leg tumor treated with BNCT and the contralateral, untreated left leg tumor for the group of BNCT-R, BNCT*, and Sham. Note that a marked tumor response to BNCT in the right leg is associated to an abscopal effect in the contralateral left leg tumor. Statistically significant abscopal effect is indicated by **a** ($p = 0.05$) and **b** ($p = 0.0013$)

may then mediate an enhanced immune response against unirradiated, malignant lesions expressing similar tumor antigens (Lakshmanagowda et al. 2009; Stamell et al. 2013). Additional evidence that a boost of the immune system induces tumor response lies in numerous studies that have shown a clear correlation between the use of microorganisms and subsequent tumor regression or a decrease or delay in the risk of cancer recurrence. The use of Bacillus Calmette-Guérin vaccine for the treatment of superficial urinary bladder cancer is the most common clinical application of microorganisms for cancer treatment. Bacteria such as *Clostridium* spp., *Bifidobacterium* spp., *Lactobacillus* spp., and *Salmonella* spp. have also been used with varying success in experimental models and/or in a clinical scenario (Kucerova and Cervinkova 2016). Recent evidence that ionizing radiation induces an immunogenic tumor cell death and alters the tumor microenvironment to enhance the recruitment of anti-tumor T cells, supports the hypothesis that ionizing radiation can enhance both the priming and effector phase of the anti-tumor immune response (Dewan et al. 2009). Reduction of an immunosuppressive tumor bulk in responsive subjects and suppression of radiosensitive immune suppressor mechanisms might also contribute to an abscopal response (Ohba et al. 1998; Rees and Ross 1983). Hartford et al. (2000) demonstrated that circulating levels of the angiogenesis inhibitor endostatin significantly increased after radiotherapy in mice. Camphausen et al. (2003) and Mozdarani (2012) suggested that radiotherapy may lead to a systemic antiangiogenic effect mediated through p53, conceivably contributing to an abscopal effect.

In the present study, for the first time, proof of principle is demonstrated that BNCT alone is capable of eliciting an abscopal effect on tumor development distant from the primary site of irradiation. These abscopal effects were exclusively observed among all the rats that displayed a local response to BNCT. The need for a positive response to local radiotherapy for an abscopal response to occur has been previously described for standard radiotherapy (Grimaldi et al. 2014; Zenkoh et al. 2015). It is postulated that following radiotherapy-mediated tumor cell death, T-cell priming would occur through dendritic cell cross presentation of released tumor antigens in draining lymph nodes, leading to rejection of the primary or metastatic tumors (Park et al. 2014).

It is well known that radiation effects on tumors depend on the dose per fraction applied, the number of fractions used, and the total dose. The type of dose fractionation regimen has been shown to determine the ability of radiotherapy to cause an abscopal effect (Camphausen et al. 2003; Dewan et al. 2009; Lugade et al. 2005). Moreover, the interplay between these variables will depend on the tumor setting (Demaria and Formenti 2012). Seeking to provide proof of principle of the abscopal effect of BNCT alone, a single treatment protocol and radiation dose prescription was explored here. Based on previous BNCT studies performed by the authors' group in an experimental model employing the same colon cancer cells (e.g., Pozzi et al. 2013), the experimental procedure employed here would be capable of eliciting a positive tumor response in the irradiated area. Different BNCT protocols (e.g., Molinari et al. 2011) might enhance the effect demonstrated in the present study and warrant evaluation. While the extrapolation of data from translational research to a clinical scenario has well-known limitations, the protocols that prove beneficial in an experimental model might have a better chance of efficacy in a clinical study as shown by Golden et al. (2013).

The use of a model based on the inoculation of syngeneic colon cancer cells in immunocompetent BDIX rats was essential to demonstrate an abscopal effect. Previous studies reported that the abscopal effect was dependent on a functional immune system (Camphausen et al. 2003; Demaria et al. 2004; Chakravarty et al. 1999), capable of

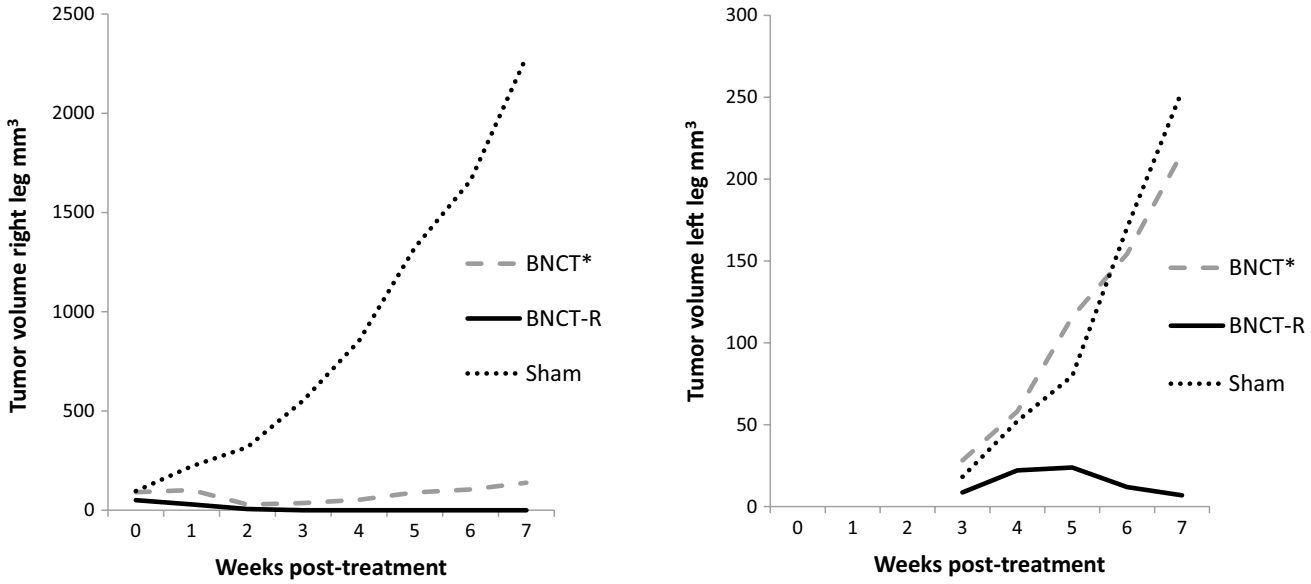


**Fig. 3** Right leg and contralateral left leg mean tumor volume as a function of time post-treatment of the right leg tumor for the different experimental groups, showing the reduction in right leg tumor volume in the BNCT-R group and the associated abscopal effect in the left leg tumor over time. The standard deviation has been omitted for the sake of clarity (individual tumor volume values are presented in Table 2)

undergoing a shift in phenotype from immune escape toward immune-mediated tumor elimination (Postow et al. 2012). In this sense, experimental models based on the inoculation of human tumor cell lines in immunodeficient animals would not be adequate for studies of abscopal effect. An abscopal effect was not observed in nude mice which lack T cells (Sham 1995). In addition, the present preliminary findings regarding some degree of association between female gender/hormonal status and abscopal effect warrant further studies. Hormonal status might conceivably influence an immune-mediated response.

Cell death induced by BNCT can occur via necrosis and/or apoptosis (e.g., Trivillin et al. 2006; Masunaga et al. 2002; Aromando et al. 2009). However, necrosis would be the most frequent mechanism (Trivillin et al. 2006; Aromando et al. 2009). The induction of death of cancer cells by necrosis will induce inflammation and the release of endogenous danger signals known as damage-associated molecular patterns that may be critical to the induction of an abscopal effect (Ludgate 2012). The predominant mechanism of cell death by BNCT would thus favor an abscopal effect. Moreover, while apoptosis was originally considered non-immunogenic and non-inflammatory, some studies have provided evidence that apoptosis is immunogenic when it is induced by ionizing radiation (Masucci et al. 2012). Thus, death by apoptosis might also contribute to the abscopal effect.

Admittedly, radiation is a complex modifier of the tumor microenvironment and, by itself, is seldom sufficient to induce a therapeutically significant anti-tumor immune response, since it can also activate immune suppressive pathways (Demaria and Formenti 2012). The present evidence of an unequivocal, robust abscopal effect of BNCT alone is particularly significant given that standard radiotherapy alone is rarely able to induce an abscopal effect (Dewan et al. 2009). Nonetheless, it would conceivably be possible to enhance this effect with immunotherapeutic interventions. Based on the effect of whole-body myeloablative irradiation regimens, local radiotherapy was erroneously considered immunosuppressive. Conversely, radiotherapy would be a tool to convert cancer cells into an anticancer vaccine in situ (Formenti and Demaria 2013; Golden and Formenti 2014). However, the immunostimulatory effects of local radiotherapy are weakened by the microenvironment of established tumors which is often highly immunosuppressive. Abnormal tumor angiogenesis plays an important role in tumor-induced immune dysregulation (Vatner et al. 2014). Immunotherapeutic strategies that contribute to overcome immune escape of cancer and recover immune-rejection (Mellman et al. 2012) would be necessary for radiotherapy to be maximally efficient (Golden and Formenti 2014). Within this context, the abscopal effect of BNCT demonstrated herein might conceivably be boosted by immunotherapy.

## Conclusions

In the present study, it was demonstrated that the use of BNCT alone produces significant local tumor control and is

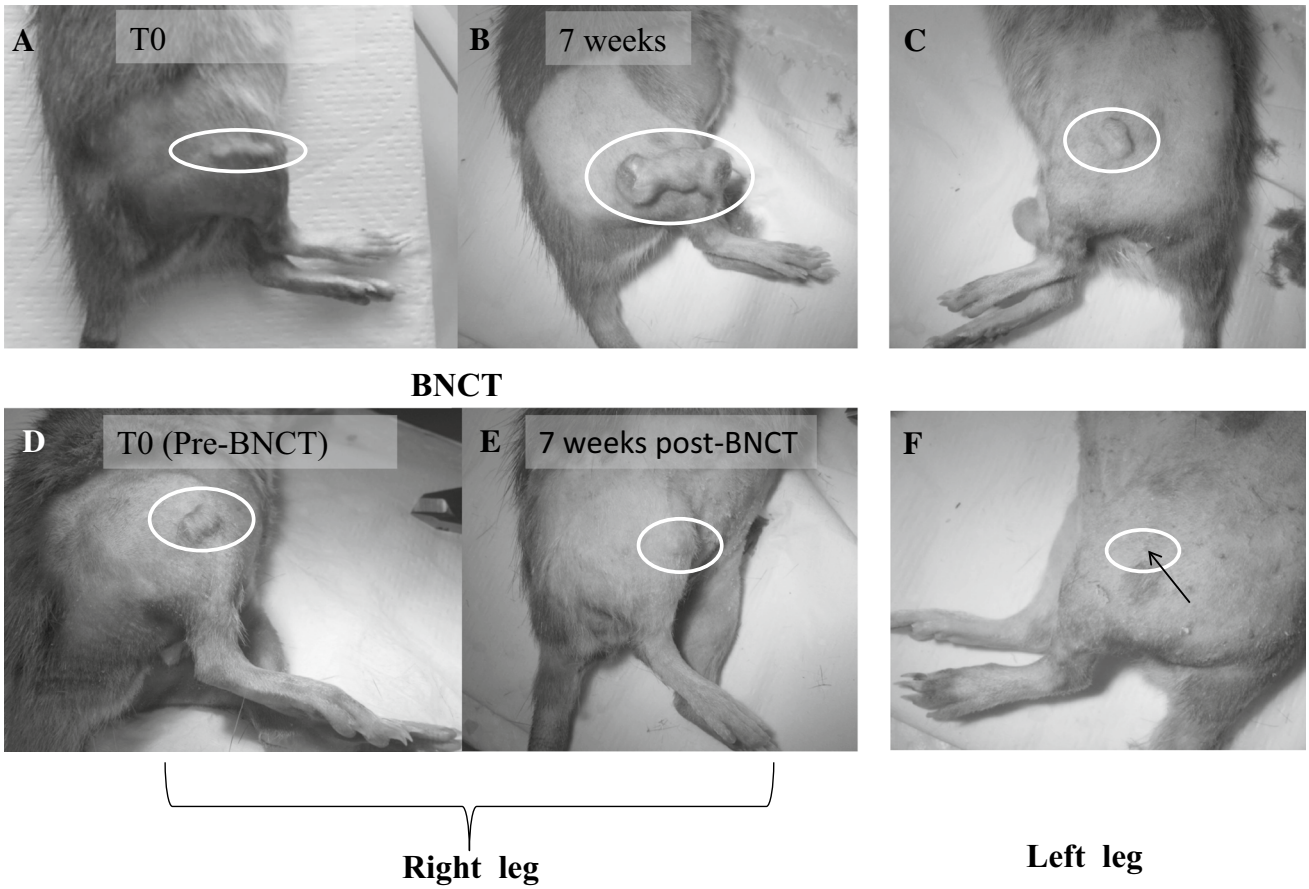


**Fig. 4** Upper panel, representative example of untreated control: **a** right leg bearing tumor 3 weeks post-inoculation (T0); **b** right leg bearing untreated tumor 7 weeks later. Tumor growth is evident; **c** contralateral left leg showing conspicuous tumor growth. *Lower panel*, representative example of BNCT-treated rat: **a** right leg bearing tumor 3 weeks post-inoculation (T0), pre-BNCT; **b** right leg bearing tumor 7 weeks post-BNCT. A reduction in tumor volume is evident indicating a positive response to BNCT; **c** contralateral left leg showing a hardly detectable tumor indicated by an *arrow*. In all cases the tumor area is encircled in *white*

also capable of inducing an anti-tumor response at a distant site. The combination of BNCT and immune-based therapeutic modalities might constitute a potentially potent synergistic approach to provide long-term protection and minimal toxicity, warranting future studies.

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**Compliance with ethical standards**

**Conflict of interest** Author VA Trivillin declares that she has no conflict of interest. Author ECC Pozzi declares that he has no conflict of interest. Author LL Colombo declares that he has no conflict of interest. Author SI Thorp declares that she has no conflict of interest. Author MA Garabalino declares that she has no conflict of interest. Author A Monti Hughes declares that she has no conflict of interest. Author SJ González declares that she has no conflict of interest. Author RO Farías declares that he has no conflict of interest. Author P Curotto declares that she has no conflict of interest. Author GA Santa Cruz declares that he has no conflict of interest. Author DG Carando declares that he has no conflict of interest. Author AE Schwint declares that she has no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Human and animal rights** This article does not contain any studies with human participants performed by any of the authors.

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