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ORIGINAL PAPER

Boron neutron capture therapy (BNCT) for liver metastasis in an experimental model: dose–response at five-week follow-up based on retrospective dose assessment in individual rats

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Abstract Boron neutron capture therapy (BNCT) was proposed for untreatable colorectal liver metastases. Employing an experimental model of liver metastases in rats, we recently demonstrated that BNCT mediated by boronophenylalanine (BPA-BNCT) at 13 Gy prescribed to tumor is therapeutically useful at 3-week follow-up. The aim of the present study was to evaluate dose–response at 5-week follow-up, based on retrospective dose assessment in individual rats. BDIX rats were inoculated with syngeneic colon cancer cells DHD/K12/TRb. Tumor-bearing animals were divided into three groups: BPA-BNCT ($n = 19$), Beam only ($n = 8$) and Sham ($n = 7$) (matched manipulation, no treatment). For each rat, neutron flux was measured in situ and boron content was measured in a pre-irradiation blood sample for retrospective individual dose assessment. For statistical analysis (ANOVA), individual data for the BPA-BNCT group were pooled according to absorbed tumor dose, BPA-BNCT I: 4.5–8.9 Gy and BPA-BNCT II: 9.2–16 Gy. At 5 weeks post-irradiation, the tumor surface area post-treatment/pre-treatment ratio was 12.2 ± 6.6 for Sham, 7.8 ± 4.1 for Beam only, 4.4 ± 5.6 for BPA-BNCT I and 0.45 ± 0.20 for BPA-BNCT II; tumor nodule weight was 750 ± 480 mg for Sham, 960 ± 620 mg for Beam only, 380 ± 720 mg for BPA-BNCT I and 7.3 ± 5.9 mg for BPA-BNCT II. The BPA-BNCT II group exhibited statistically significant tumor control with no contributory liver toxicity. Potential threshold doses for tumor response and significant tumor control were established at 6.1 and 9.2 Gy, respectively.

Keywords Boron neutron capture therapy · BNCT · Liver metastasis · Liver metastasis experimental model · BDIX rats · DHD/K12/TRb cells

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Introduction

Boron neutron capture therapy (BNCT) is a binary treatment modality that combines irradiation with a thermal or epithermal neutron beam with tumor-seeking, boron-containing drugs to produce preferential irradiation of tumor tissue. The high linear energy transfer (LET) alpha particles (1.47 MeV) and recoiling ^{7}Li nuclei (0.84 MeV) emitted after the $^{10}B(n,\alpha)^{7}Li$ reaction, have a range of 5–9 μm in tissue and are known to have a high relative biological effectiveness (Coderre and Morris 1999). In this way, BNCT would potentially target the tumor selectively, minimizing damage to normal tissue. The radiation doses delivered to tumor and normal tissues during BNCT are due to energy deposition from directly ionizing radiation with different LET characteristics. In addition to the alpha and ^{7}Li high-LET products that give rise to the tumor-specific boron dose component, a non-specific background dose results from: (1) low-LET gamma rays in the beam, (2) low-LET gamma rays resulting from the capture of thermal neutrons by hydrogen atoms [$^{1}H(n,\gamma)^{2}H$], (3) high-LET protons produced by the scattering of fast neutrons when a hardened epithermal neutron beam spectrum is employed and (4) high-LET protons resulting from the capture of thermal neutrons by nitrogen atoms [$^{14}N(n,p)^{14}C$]. The biologically effective dose will depend on relative biological effectiveness (RBE) and compound biological effectiveness (CBE) factors (Coderre and Morris 1999) for the different dose components in each case. BNCT protocols are ideally designed to maximize the boron radiation dose and to minimize the background dose (e.g., Coderre and Morris 1999; Kreimann et al. 2001; Trivillin et al. 2006; Molinari et al. 2011, 2012). Furthermore, being a technique that is based on biochemical targeting rather than geometric targeting, it would be ideally suited to treat undetectable micrometastases, a major challenge in oncological therapy (e.g., Cardoso et al. 2007).

Clinical trials of BNCT for the treatment for glioblastoma multiforme and/or melanoma and, more recently, head and neck tumors, using boronophenylalanine (BPA) or sodium mercaptoundecahydrododecaborane (BSH) as the boron carriers, have been performed or are underway in Argentina, the European consortium, Finland, Japan, Sweden, Taiwan and the United States (e.g., Chanana et al. 1999; Busse et al. 2003; Diaz 2003; Gonzalez et al. 2004; Kankaanranta et al. 2011, 2012; Nakai et al. 2011; Wang et al. 2011; Aiyama et al. 2011). Translational studies in appropriate experimental models have advanced the knowledge of BNCT radiobiology and contributed to the optimization of this technique for different tumor types and sites (e.g., Trivillin et al. 2008; Barth et al. 2012; Heber et al. 2012; Garabalino et al. 2013).

Multifocal, non-resectable liver metastases from colorectal cancer that do not respond to chemotherapy are a potential target for BNCT. A treatment option in these cases is particularly beneficial because the primary tumor in the colon can generally be successfully excised, and liver is frequently the only site of metastatic spread (Nano et al. 2004). Ex situ BNCT mediated by BPA, followed by whole liver autograft reportedly controlled metastatic liver nodules in two treated patients (Zonta et al. 2006). Some boron biodistribution studies have been performed in liver tumor experimental models (e.g., Pinelli et al. 2001; Roveda et al. 2004; Suzuki et al. 2004; Liao et al. 2010) and in liver metastasis patients (Altieri et al. 2004; Wittig et al. 2008; Cardoso et al. 2009). Some attempts have also been made to perform BNCT studies in experimental models employing end points related indirectly to liver tumor control (Nano et al. 2004; Suzuki et al. 2000). Studies in an experimental liver metastases model contribute to the understanding of BNCT radiobiology for this pathology and to the optimization of the design of therapeutically useful, safe clinical BNCT protocols. We first performed boron biodistribution studies with 11 administration protocols employing the boron compounds BPA and decahydrodecaborate (GB-10) in a liver metastasis model in BDIX rats that allows for the evaluation of boron content in blood, tumor tissue and a wide variety of potentially dose-limiting healthy tissues (Garabalino et al. 2011). Based on this biodistribution study, we selected one of the potentially useful boron compound administration protocols [BPA at a dose of 46.5 mg ^{10}B/kg bm, intraperitoneally (ip) + intravenously (iv)] and performed the first systematic in vivo BNCT study in an experimental liver metastases model in BDIX rats to assess tumor control and potential radiotoxicity at 3-week follow-up, at a prescribed absorbed tumor dose of 13 Gy (Pozzi et al. 2012). Having demonstrated partial remission in 100 % of the tumor nodules at 3 weeks post-treatment, the aim of the present study was to assess tumor control and normal tissue toxicity at 5 weeks post-treatment. In view of potential differences between prescribed and administered dose, retrospective dose assessment was performed in each of the animals. The working hypothesis was that the differences between prescribed and administered dose would be due to the well-known spread in boron concentration values (e.g., Garabalino et al. 2011), variations in tumor neutron flux resulting mainly from differences in positioning of the animal within the shielding device and lack of reproducibility in intraperitoneal administration of drugs (BPA in this case). In the present study, tumor neutron flux was measured in situ and pre-irradiation blood boron concentration was evaluated in each animal for retrospective dose assessment. This procedure allowed a dose–response analysis at 5 weeks post-treatment and establishment of a

potential threshold dose for tumor control. Histological analysis of tumor remnants at 5 weeks post-treatment was performed to establish degree of histological response employing a semi-quantitative scale.

Materials and methods

A total of 44 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 one per cage in a room with controlled temperature and humidity with 12-h light/dark cycles. Following laparotomy under ketamine (36.5 mg/kg bm)—xylazine (5.4 mg/kg bm) anesthesia, subcapsular inoculation of 5×10^5 syngeneic colon cancer cells, DHD/K12/TRb (ECACC, UK), in 10 µl of F10-DMEM culture medium (GIBCO) was performed in the left lateral lobe of the liver using a Hamilton syringe with a 22-gauge needle. The experimental liver metastasis model was adapted from Roveda et al. (2004) and Caruso et al. (1993). Subcapsular inoculations were performed to induce the development of subcapsular tumor nodules that simulate liver metastases and are more amenable to follow-up (De Jong et al. 2009). Two weeks post-inoculation, 100 % of the animals developed localized, measurable, vascularized tumor nodules, with no peritoneal or pulmonary dissemination as previously described (Pozzi et al. 2012).

Two weeks post-inoculation, tumor-bearing animals were used for in vivo BNCT studies at the RA-3 nuclear reactor thermal facility described elsewhere (Miller et al. 2009). A lithium carbonate shield (enriched to 95 % in ^{6}Li), fabricated ad hoc, was used to protect the body of the animal while exposing the liver area through a window (Pozzi et al. 2012). Due to the high boron content of the kidney at the time of irradiation (Garabalino et al. 2011), acrylic tabs were used to artificially distance the kidneys from the window during irradiation and thus minimize kidney radiotoxicity. Dosimetric calculations were based on previously reported physical dosimetry data for the RA-3 facility (Pozzi et al. 2009). In this facility, the neutron field is very well thermalized, making the radiation dose component from hydrogen recoil (i.e., fast neutron dose) in tissue negligible. Reported absorbed dose includes three components: a proton dose from nitrogen capture (with thermal neutron kerma factors for each body tissue taken from ICRU46 1992), a gamma dose and a boron contribution reported as Gy per part per million boron by mass. In order to obtain boron dose, this last contribution should be multiplied by the corresponding boron concentration. Boron biodistribution data in this model were reported previously (Garabalino et al. 2011) and used for dose prescription. Self-powered neutron detector (SPND) (Miller et al. 2004) measurements at a monitor position were performed during each irradiation to check for potential variations in the neutron flux and estimate the exposure time to reach the prescribed dose in each case. Gamma dose rate was previously measured with a graphite ionization chamber neutron shielded with a LiF cap (95 % enriched in ^{6}Li) and was assumed homogeneous for all tissues.

Tumor-bearing animals were divided up at random into three groups: BPA-BNCT ($n = 19$): BPA (L-enantiomer, >98 % enriched in ^{10}B, Boron Biologicals, Inc., Raleigh, NC, USA) 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 intraperitoneally (ip) in 17 rats and (ip + intravenously [iv]) in two rats at a dose of 46.5 mg ^{10}B/kg body mass (bm). The boron compound administration protocols were selected based on the previously reported boron biodistribution data. Both protocols complied with the established guidelines for potential therapeutic value, i.e., no manifest toxicity, absolute boron concentration in tumor >20 ppm, boron concentration ratio tumor/normal liver ≥1 (Garabalino et al. 2011). Three hours post-administration, the animals were exposed to neutron irradiation; Beam only ($n = 8$): no BPA administration exposed to the same neutron fluence as the BPA-BNCT group to assess the effect of background dose; Sham ($n = 7$): control group exposed to matched manipulation (tumor cell inoculation and laparoscopy 2 weeks post-inoculation) to simulate treatment, but left untreated (same manipulation but no boron compound administration and no exposure to neutrons).

Since no noninvasive technique is available to us to date to follow tumor growth in the rat liver, prior to irradiation the animals were submitted to laparotomy to measure the pre-treatment surface area of the tumor nodule. Two very thin copper wires, 0.25 mm in diameter and 7 mm in length, were inserted in the liver, alongside the tumor nodule, in all animals to assess the flux in the tumor site and its ratio to flux measured at the external SPND monitor site. In addition, in the BPA-BNCT group, the peritoneal cavity was flushed with warm saline solution to remove residual BPA in the intraperitoneal liquid that could give rise to boron neutron capture reactions and contribute to intestinal radiotoxicity. Furthermore, prior to irradiation, blood samples were taken for boron measurement at a later date. The animals were loosely sutured prior to irradiation. After irradiation, the abdomen was reopened to remove the copper wires and re-sutured. The wires were measured by gamma spectrometry to obtain the thermal neutron flux in the tumor area. Gamma rays from the activated wires were measured using a high-purity germanium detector (HPGe) previously calibrated using a commercial Europium

(^{152}Eu) source of certified activity (Certificate of calibration No. 76044A-440, Eckert & Ziegler Analytics, Inc., October 30, 2007. Analytics maintain traceability to National Institute of Standards and Technology). As the wire material was pure copper (99.95 %), only the 511 keV gamma ray from the ^{63}Cu(n,γ)^{64}Cu reaction, with a cross section of 4.5 barns for 0.0253 eV neutrons, was considered. From previous measurements (Miller et al. 2009), flux can be assumed as pure thermal and contributions from epithermal neutrons to activation can be considered negligible. The wires were measured at the greatest distance from detector available in order to fulfill the condition of point source. Efficiency for the 511 keV activation peak in the chosen position was 0.000891 (±5 %) cps/γ, making it possible to obtain a total number of counts with uncertainties not exceeding 1 % in reasonable times. The blood samples were processed as previously described (Garabalino et al. 2011; Heber et al. 2012) for boron assessment by atomic emission spectroscopy with inductively coupled plasma (ICP-OES Optima 3100 XL, UV, axial, Perkin Elmer) or inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC2, Perkin Elmer).

In the BPA-BNCT group, the thermal neutron fluence was prescribed, to deliver a total absorbed dose range to tumor of 6–15 Gy. Estimation of the boron dose component was based on previously reported tumor boron concentration values (Garabalino et al. 2011). The best estimation of the tumor dose delivered to each animal was retrospectively determined based on the thermal neutron flux in the tumor area and the tumor boron concentration inferred from the actual blood boron concentration at the time of irradiation. The tumor/blood ratio of boron concentration employed to estimate tumor boron concentration from blood boron concentration was taken from previously reported biodistribution data for the BPA administration protocol employed herein (Garabalino et al. 2011). Assuming that normal liver surrounding tumor was exposed to the same neutron flux as tumor, the dose to normal liver was retrospectively estimated from measured blood boron concentration in each case and previously reported boron concentration ratios (Garabalino et al. 2011). The dose quoted for normal liver corresponds to only a portion of the liver and must not be interpreted as the dose delivered to the whole organ (due to the shielding device, a large proportion of the liver was exposed to a lower neutron flux). The dose quoted for the rest of the organs corresponds to the doses associated with the prescribed tumor dose.

The average thermal neutron flux in the inoculated liver lobe, obtained from activation of the copper wires inserted alongside the tumor, was $(2.3 \pm 0.5) \times 10^9$ n cm^{-2} s^{-1}, where the main contribution to uncertainty comes from statistical dispersion of data. Individual blood boron values ranged from 1.2 to 16 ppm. The actual total estimated absorbed dose administered with BPA-BNCT ranged from 4.5 ± 0.3 to 16 ± 4 Gy to tumor and 4.2 ± 0.4 to 11 ± 2 Gy to normal liver. The boron dose component ranged from 0.7 ± 0.2 to 12 ± 4 Gy to tumor and 0.5 ± 0.2 to 7 ± 2 Gy to normal liver. In all cases, the Beam only dose 3.6 ± 0.3 Gy corresponded to exposure to the higher neutron fluence range, prescribed to the BPA-BNCT group.

The animals were followed for 5 weeks post-treatment. During that time, clinical signs and body mass were monitored regularly. At the end of the experiment (5 weeks post-treatment), the animals were killed and tumor nodule surface area was re-measured. In addition, dissected tumor nodules were weighed. Samples of remaining tumor nodule (when large enough to permit sampling) and of normal liver were taken for histological analysis of tumor response and potential liver toxicity, respectively. An additional set of tumor-bearing animals ($n = 10$) were killed 2 weeks post-inoculation to determine mean tumor weight before treatment (T0).

The tumor response end points evaluated were post-treatment/pre-treatment tumor nodule surface area ratio and tumor nodule mass. The end point Post/Pre Area can be calculated individually for each animal. Although this is of course an advantage, this end point is only an indirect measure of tumor volume/mass. Conversely, post-treatment tumor nodule mass is a direct measure of tumor volume/mass but can only be compared to *mean* pre-treatment tumor nodule mass. Measurement of actual pre-treatment tumor nodule mass for each animal is naturally not possible. Thus, both end points were considered contributory and complementary.

In addition, when possible, a complementary assessment of histological tumor features was performed to establish the degree of histological response. A semi-quantitative scale was employed based on the following end points: ratio parenchyma/stroma, persistence of glandular differentiation, proportion of viable-looking (leptochromatic) nuclei versus damaged (bizarre, hyperchromatic, pleomorphic) nuclei and presence of mitosis. Histological response for each rat was graded as low, intermediate or high based on subjective screening of hematoxylin-eosin stained sections by a single, trained observer. In the cases in which the remaining tumor was not large enough for sampling for histological evaluation, response was considered high.

Radiotoxicity was evaluated in terms of clinical signs, body mass changes, skin healing and macroscopic/histological alterations in normal liver.

When relevant, statistical analysis was performed by analysis of variance (ANOVA). Statistical significance was set at $p = 0.05$.

Animal care was in accordance with current laws of Argentina and institutional guidelines. The studies were

reviewed and approved by the committee of the National Atomic Energy Commission (Argentina) that oversees the ethics of research involving animals (Approval CICUAL-CNEA/2011).

Results

The post-treatment/pre-treatment tumor nodule surface area ratio (Post/Pre Area) and tumor nodule mass 5 weeks after BPA-BNCT for each of the irradiated animals are shown in Table 1. Estimated total absorbed dose and boron dose component delivered to tumor and normal liver, estimated retrospectively as previously described, are shown in each case. Table 2 presents, for each organ/tissue, the mean thermal neutron fluence, the absorbed doses from the different radiation components and the corresponding total absorbed background dose, for the irradiated animals.

Figures 1 and 2 show individual rat tumor response in terms of the end points Post/Pre Area and tumor nodule

Table 1 Values of end points and doses calculated retrospectively for each animal treated with BPA-BNCT

# Rat	Absorbed dose (Gy)				End point	
	Normal liver		Tumor		Tumor	
	Total dose	Boron dose	Total dose	Boron dose	Post/Pre Area	Mass (mg)
R66	4.1 ± 0.4	0.3 ± 0.3	4.5 ± 0.3	0.7 ± 0.2	17	2,200
R50	4.2 ± 0.4	0.5 ± 0.3	4.8 ± 0.4	1.1 ± 0.3	13	1,140
R4	5.1 ± 0.5	0.8 ± 0.7	6.1 ± 0.6	1.8 ± 0.5	4.0	270
R82	5.0 ± 0.8	1.4 ± 0.7	6.8 ± 0.9	3.3 ± 0.9	3.1	55
R81	5.0 ± 0.8	1.5 ± 0.7	6.9 ± 1.0	3.5 ± 0.9	0.34	15.2
R73	5.5 ± 1.0	2.0 ± 1.0	8.1 ± 1.3	4.7 ± 1.2	1.7	28
R83	5.6 ± 1.0	2.1 ± 1.0	8.3 ± 1.3	4.8 ± 1.3	0.88	29
R95	6.2 ± 1.0	2.0 ± 1.0	8.8 ± 1.3	4.6 ± 1.2	0.95	59
R94	6.2 ± 1.0	2.0 ± 1.0	8.8 ± 1.3	4.7 ± 1.2	1.2	60
R61	6.9 ± 0.9	2.5 ± 0.9	8.9 ± 1.5	4.5 ± 1.5	2.3	16.7
R77	6.0 ± 1.2	2.4 ± 1.2	9.2 ± 1.5	5.6 ± 1.5	0.33	3.3
R76	6.1 ± 1.2	2.5 ± 1.2	9.4 ± 1.5	5.8 ± 1.5	0.31	4.9
R51	6.6 ± 1.5	3.0 ± 1.5	10.5 ± 1.8	6.9 ± 1.8	0.65	7.7
R86	7.0 ± 1.4	2.8 ± 1.4	10.6 ± 1.7	6.3 ± 1.7	0.69	21
R80	6.8 ± 1.5	3.1 ± 1.5	10.9 ± 1.9	7.2 ± 1.9	0.55	8.2
R85	7.3 ± 1.5	3.0 ± 1.5	11.2 ± 1.8	7 ± 2	0.22	5.6
R60	7.0 ± 1.6	3.3 ± 1.6	11 ± 2	7.5 ± 1.8	0.25	0.9
R84	7.7 ± 1.7	3.4 ± 1.7	12 ± 2	8 ± 2	0.73	10.4
R62	11 ± 2	7 ± 2	16 ± 4	12 ± 4	0.32	3.6

The uncertainty associated with Post/Pre Area values was estimated at 10 %, and the uncertainty associated with mass values was estimated at 5 %

Table 2 Absorbed dose radiation components for each tissue calculated as mean ± standard deviation over the 19 irradiated animals

Tissue	Fluence (n cm^{-2})	Gamma photons (Gy)	Induced protons (N^{14}) (Gy)	Boron (Gy per μg g^{-1})[a]	Total absorbed background dose (Gy)
Tumor	$(3.3 \pm 0.8) \times 10^{12}$	3.2 ± 0.3	0.69 ± 0.16	0.25 ± 0.06	3.9 ± 0.4
Normal liver[b]	$(3.3 \pm 0.8) \times 10^{12}$	3.2 ± 0.3	0.69 ± 0.16	0.25 ± 0.06	3.9 ± 0.4
Skin[c]	$(6.7 \pm 0.6) \times 10^{12}$	3.2 ± 0.3	1.95 ± 0.16	0.50 ± 0.04	5.2 ± 0.4
Kidney	$(1.01 \pm 0.08) \times 10^{12}$	3.2 ± 0.3	0.21 ± 0.02	0.075 ± 0.006	3.4 ± 0.3
Intestine	$(1.01 \pm 0.08) \times 10^{12}$	3.2 ± 0.3	0.156 ± 0.013	0.075 ± 0.006	3.4 ± 0.3
Lung	$(2.0 \pm 0.2) \times 10^{12}$	3.2 ± 0.3	0.43 ± 0.04	0.150 ± 0.012	3.6 ± 0.3

[a] Boron dose is quoted as Gy per part per million boron by mass

[b] Normal liver surrounding tumor nodule

[c] Non-shielded skin

mass, respectively, plotted as a function of absorbed dose. Considering the end point tumor nodule mass and the 95 % confidence interval (CI) of the values for the Sham group [310–1,190 mg] and for the Beam only group [450–1,470 mg], rats 66 and 50 were considered non-responsive because their corresponding tumor nodule mass value fell within or above the 95 % CI for both the Sham and the Beam only groups. Similarly, considering the end point Post/Pre Area and the 95 % CI of the values for the Sham group [6.1–18] and for the Beam only group [4.4–11], rats 66 and 50 were considered non-responsive. The remaining rats were considered responsive. The lowest total dose at which tumor response was evidenced for both end points was 6.1 Gy (Rat 4). These findings would suggest a potential dose threshold for some degree of tumor response at about 6.1 Gy and a boron dose of about 1.8 Gy.

For the sake of statistical analysis, data for the BPA-BNCT group were pooled separately for BPA-BNCT I: total absorbed dose to tumor of 4.5–8.9 Gy (tumor boron dose between 0.7 and 4.5 Gy) and BPA-BNCT II: total absorbed dose to tumor of 9.2–16 Gy (tumor boron dose between 5.6 and 11.8 Gy). The cutoff dose value between both groups was chosen subjectively based on degree of response in terms of both the end points evaluated. Table 3 shows the data pooled for BPA-BNCT I, BPA-BNCT II, Beam only and Sham groups. At 5 weeks, Post/Pre Area was 12.2 ± 6.6 for the Sham group. This value indicates that, left untreated, tumor surface area increased on average approximately 12 times over a 5-week period. Post/Pre Area was 7.8 ± 4.1 for the Beam only group. The difference between the Sham and Beam only groups was not statistically significant. In the case of BPA-BNCT I, Post/Pre Area was 4.4 ± 5.6. This value was significantly smaller than the corresponding value for the Sham group ($p = 0.019$), but was not significantly different from the Beam only group. In the case of BPA-BNCT II, Post/Pre

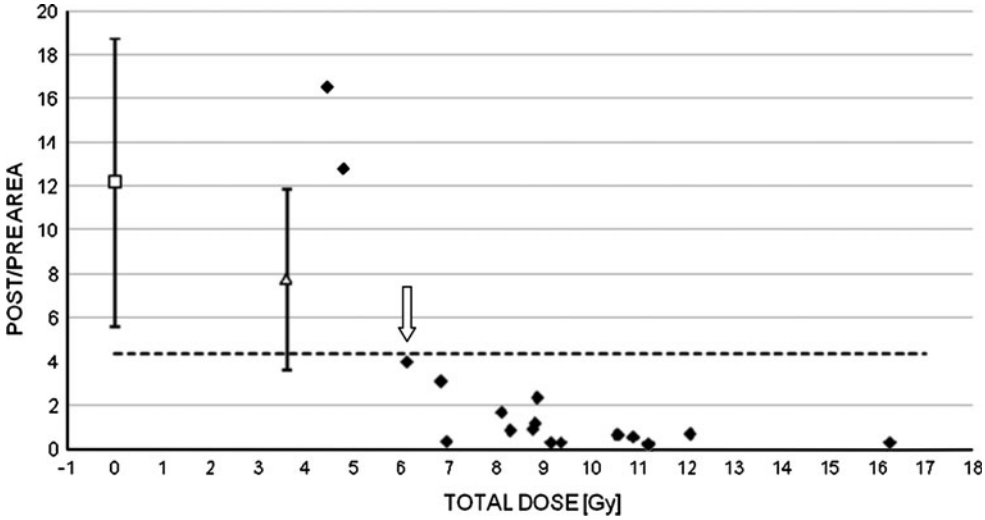

Fig. 1 Individual rat tumor response in terms of the end point Post/Pre Area plotted as a function of time. (*open square*)—Sham (mean ± standard deviation); (*open triangle*)—Beam only (mean ± standard deviation); (*filled diamond*)—BPA-BNCT; *dashed line*—threshold for some degree of tumor response; the *arrow* indicates threshold dose for some degree of tumor response

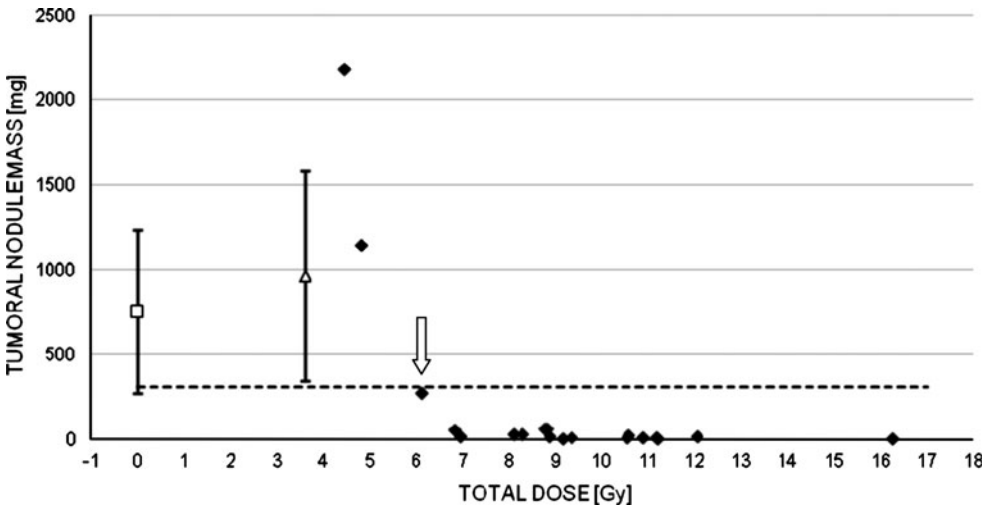

Fig. 2 Individual rat tumor response in terms of the end point tumor nodule mass plotted as a function of time. (*open square*)—Sham (mean ± standard deviation); (*open triangle*)—beam only (mean ± standard deviation); (*filled diamond*)—BPA-BNCT; *dashed line*—threshold for some degree of tumor response; the *arrow* indicates threshold dose for some degree of tumor response

Table 3 Pooled data for each of the groups and end points evaluated

Treatment	Post-/pre-treatment surface area ratio	Tumor nodule mass post-treatment (mg)
Sham	12.2 ± 6.6	750 ± 480
	$n = 7$	$n = 7$
Beam only	7.8 ± 4.1	960 ± 620
	$n = 8$	$n = 8$
BPA-BNCT I	4.4 ± 5.6[a]	390 ± 720
	$n = 10$	$n = 10$
BPA-BNCT II	0.45 ± 0.20[b]	7.3 ± 5.9[c]
	$n = 9$	$n = 9$

[a] Statistically significant difference versus Sham

[b] Statistically significant difference versus BPA-BNCT I, beam only and Sham

[c] Statistically significant difference versus beam only and Sham

Area was 0.4 ± 0.2. This value was significantly smaller than for the Sham group ($p = 0.000$), the Beam only group ($p = 0.001$) and the BPA-BNCT I group ($p = 0.048$).

Tumor nodule mass pre-treatment was 34 ± 17 mg. At 5 weeks, tumor nodule mass rose significantly ($p = 0.0002$) to 750 ± 480 mg for the Sham group and to 960 ± 620 for the Beam only group and rose (albeit not significantly) to 390 ± 720 mg for the BPA-BNCT I group and fell significantly ($p = 0.0003$) to 7.3 ± 5.9 mg for the BPA-BNCT II group. No statistically significant differences were found between the Sham and Beam only groups or between the BPA-BNCT I group and either of the Sham or Beam only group. Conversely, highly statistically significant differences were found between the BPA-BNCT II group and the Beam only and Sham groups ($p = 000$). Although the means corresponding to the BPA-BNCT I and BPA-BNCT II groups differed widely (390 ± 720 vs 7.3 ± 5.9, respectively), the difference did not reach statistical significance, conceivably due to the wide spread in values. Importantly, BPA-BNCT II achieved a 99 % reduction (1–[7.3/750]) in tumor mass compared to untreated tumors (Sham group), whereas BPA-BNCT I achieved only a 49 % reduction (1–[390/750]).

To summarize, the analysis of the pooled data showed a statistically significant response (versus Sham and Beam only groups) for BPA-BNCT II. Tumor response did not reach statistical significance for BPA-BNCT I, suggesting a threshold total dose of about 9.2 Gy (Boron dose 5.6 Gy) for statistically significant tumor control in this experimental model.

No clinical, macroscopic or histological liver toxicity was observed within the study period. However, some cases of hair loss and impaired wound healing post-suture were observed in the exposed area of animals in the BPA-BNCT II group.

Table 4 Histological grade assigned to tumor response for each rat available for evaluation (in 3 out of 19 cases technical flaws made evaluation unreliable); tumor response is given on a semi-quantitative scale based on subjective screening of hematoxylin-eosin stained sections for the end points ratio parenchyma/stroma, persistence of glandular differentiation, proportion of viable-looking (leptochromatic) nuclei versus damaged (bizarre, hyperchromatic, pleomorphic) nuclei, and presence of mitosis (see also Fig. 3)

# Rat	Total tumor dose (Gy)	Histological grade tumor response
R66	4.5 ± 0.3	Low
R50	4.8 ± 0.4	Low
R4	6.1 ± 0.6	Intermediate
R82	6.8 ± 0.9	Low
R81	6.9 ± 1.0	Low
R73	8.1 ± 1.3	Intermediate
R83	8.3 ± 1.3	Intermediate
R95	8.8 ± 1.3	Intermediate
R94	8.8 ± 1.3	Intermediate
R61	8.9 ± 1.5	Intermediate
R77	9.2 ± 1.5	High
R76	9.4 ± 1.5	High
R51	10.5 ± 1.8	High
R80	10.9 ± 1.9	High
R60	11.2 ± 1.8	High
R62	16 ± 4	High

Table 4 shows the histological grade assigned to tumor response for each rat available for evaluation (in 3 out of 19 cases technical flaws made evaluation unreliable). The animals that received the lower dose levels exhibited low histological response with tumor features that resembled those of the Sham group, whereas the animals that received the higher dose levels exhibited high histological response (Fig. 3). Histological grading correlated with the macroscopic end points evaluated.

Discussion and conclusion

The present study showed that BPA-BNCT induced a consistent, significant partial remission of experimental, implanted colorectal tumor nodules in the liver 5 weeks post-irradiation with no detectable liver toxicity within the study period. Retrospective individual dose assessment allowed for the evaluation of tumor response as a function of the actual absorbed dose delivered. In this way, it was possible to establish a potential total absorbed dose threshold for some degree of tumor response at about 6.1 Gy (boron dose of about 1.8 Gy) and a potential total absorbed dose threshold for statistically significant tumor control at about 9.2 Gy (boron dose of about 5.6 Gy). Tumor control progressed from the lower dose range to the

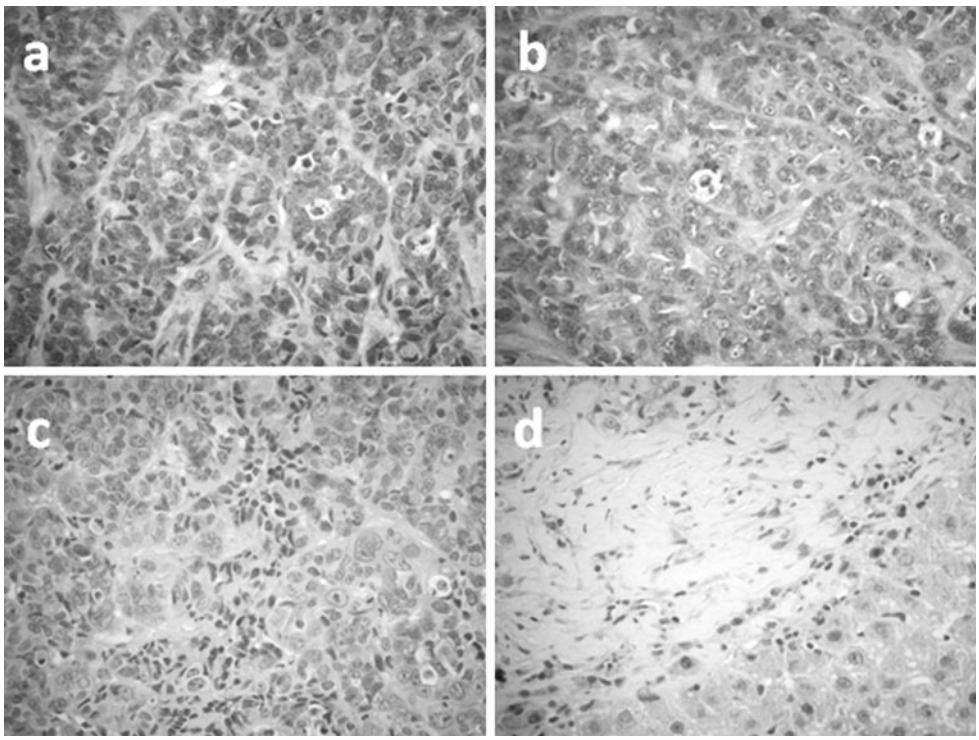

Fig. 3 Representative examples of microphotographs of histological sections corresponding to the groups **a** T0 or pre-treatment: well-differentiated adenocarcinoma with compact glandular formations and mitotic figures **b** Sham: adenocarcinoma with scarce stroma and some vacuolated nuclei, **c** BPA-BNCT, low response: persistence of glandular formations, swollen and vacuolated nuclei and scarce stroma, and **d** BPA-BNCT, high response: extensive areas of fibrohyaline stroma with remains of tumor parenchyma (cell cords with pycnotic nuclei or groups of cells with vacuolated nuclei and cytoplasmic vacuoles), flanked by normal liver. Original magnification (×400)

higher dose range, without detectable liver toxicity within the study period. However, potential long-term liver toxicity and exposure of the whole organ to higher dose ranges must be considered in a clinical scenario.

A comparison of the data reported herein for the Sham, Beam only and BPA-BNCT II groups at 5-week follow-up with previously reported data (3-week follow-up) (Pozzi et al. 2012) is shown in Table 5. In the previous 3-week follow-up study (Pozzi et al. 2012), the tumor dose was 13 ± 3 Gy. This dose range overlaps with the BPA-BNCT II dose range in the present study. Post/Pre Area increased significantly from three to 5 weeks for the Sham ($p = 0.0021$) and Beam only ($p = 0.0027$) groups but remained the same for the BPA-BNCT II group. Likewise, tumor mass increased significantly for the Sham ($p = 0.032$) and Beam only ($p = 0.0008$) groups but decreased significantly for the BPA-BNCT II group ($p = 0.049$). The tumor mass % ratio for BPA-BNCT II/Sham was 1 % at 5 weeks, falling from 6 % at 3-week follow-up. Table 6 shows the incidence of partial tumor response and of partial tumor response to less than 50 % of initial tumor surface area for each of the groups in this study. Sham and Beam only groups showed no tumor response, defining response as some degree of reduction from initial surface area. In the higher dose range group, all tumors responded whereas in the lower dose range group 30 % of the tumors responded.

Metastases of colorectal carcinoma occur most commonly in the liver (Robertson et al. 2009), and their treatment continues to pose a challenge. Although complete resection of the metastases offers the best prognosis with a 5-year survival rate of 25–45 % (Malafosse et al.

Table 5 End points for the different groups and follow-up times as indicated

Protocol/time	Three weeks post-treatment (Pozzi et al. 2012)[b]		Five weeks post-treatment (this study)	
	Post/Pre Area	Mass (mg)	Post/Pre Area	Mass (mg)
Sham	4.5 ± 3.1 ($n = 13$)	350 ± 300 ($n = 13$)	12.2 ± 6.6 ($n = 7$)	750 ± 480 ($n = 7$)
Beam only	2.7 ± 1.8 ($n = 10$)	150 ± 100 ($n = 10$)	7.8 ± 4.1 ($n = 8$)	960 ± 620 ($n = 8$)
BPA-BNCT[a]	0.47 ± 0.20 ($n = 10$)	19 ± 16 ($n = 10$)	0.45 ± 0.20 ($n = 9$)	7.3 ± 5.9 ($n = 9$)

[a] In the case of this study, data are quoted for the BPA-BNCT II group

[b] Tumor dose: 13 ± 3 Gy

Table 6 Incidence of partial tumor response (PR) and partial tumor response to less than 50 % of initial tumor surface area ($PR_{0.5}$)

Sham	Beam only	BPA-BNCT I		BPA-BNCT II	
PR	PR	PR	$PR_{0,5}$	PR	$PR_{0,5}$
0 %	0 %	30 %	10 %	100 %	56 %

2001; Fiorentini et al. 2001), only 10–15 % of all patients are eligible for surgery due to the size, number or location of the metastases (Bentrem et al. 2005). However, over the last 5 years preoperative, neoadjuvant, combination chemotherapy regimens have been reported to facilitate the downsizing of colorectal liver metastases and render initially unresectable metastases resectable (Nordlinger et al. 2007). An additional problem is the high incidence of local recurrence after surgery due to residual microscopic disease (Ruan and Warren 2005) that would cause up to 40 % of the patients to recur solely in the liver after surgical resection (Kavolius et al. 1996; Cardoso et al. 2007). In this sense, BNCT offers a mechanism to target undetectable liver micrometastases, whereas with conformal external radiotherapy, only visible liver tumors that are delineated by the physician in the treatment planning can be treated. In addition, BNCT can treat multiple liver tumors without exceeding normal liver tolerance, whereas when 3D conformal radiotherapy is applied to the treatment for more than three liver tumors, the risk of liver failure is a significant concern (Suzuki et al. 2007).

The extrapolation of translational studies to a clinical scenario is characteristically limited. In this sense, the present study affords data on the therapeutically useful tumor doses calculated from retrospectively measured boron concentration and thermal neutron flux at the tumor site. However, the experimental model does not allow analysis of the complexities of neutron flux distribution in a human liver in the case of ex situ (Zonta et al. 2006) or in situ BNCT (e.g., Suzuki et al. 2007). The challenge of achieving a homogeneous and therapeutically useful thermal neutron flux in a human liver must be addressed in each case, using strategies such as irradiation with epithermal neutron beams, use of tissue buildup material, organ rotation in the case of ex situ BNCT, multifield treatment and double application of BNCT among others.

Within this context, BNCT would be a potentially attractive technique to treat multifocal, non-resectable, bilobar liver metastases from colorectal cancers that do not respond to chemotherapy (e.g., Zonta et al. 2006; Pozzi et al. 2012). The present study presents unequivocal evidence of the therapeutic efficacy of BNCT for liver metastases with no detectable liver toxicity in an experimental model and provides radiobiological data that would be pivotal to designing potentially useful clinical protocols.

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Ethical standards The experiments comply with the current laws of Argentina.

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