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ORIGINAL PAPER

Boron delivery with liposomes for boron neutron capture therapy (BNCT): biodistribution studies in an experimental model of oral cancer demonstrating therapeutic potential

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Abstract Boron neutron capture therapy (BNCT) combines selective accumulation of ^{10}B carriers in tumor tissue with subsequent neutron irradiation. We previously demonstrated the therapeutic efficacy of BNCT in the hamster cheek pouch oral cancer model. Optimization of BNCT depends largely on improving boron targeting to tumor cells. Seeking to maximize the potential of BNCT for the treatment for head and neck cancer, the aim of the present study was to perform boron biodistribution studies in the oral cancer model employing two different liposome formulations that were previously tested for a different pathology, i.e., in experimental mammary carcinoma in BALB/c mice: (1) MAC: liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer membrane and encapsulating a hypertonic buffer, administered intravenously at 6 mg B per kg body weight, and (2) MAC-TAC: liposomes incorporating K[*nido*-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer membrane and encapsulating a concentrated aqueous solution of the hydrophilic species Na_3 [*ae*-$B_{20}H_{17}NH_3$], administered intravenously at 18 mg B per kg body weight. Samples of tumor, precancerous and normal pouch tissue, spleen, liver, kidney, and blood were taken at different times post-administration and processed to measure boron content by inductively coupled plasma mass spectrometry. No ostensible clinical toxic effects were observed with the selected formulations. Both MAC and MAC-TAC delivered boron selectively to tumor tissue. Absolute tumor values for MAC-TAC peaked to 66.6 ± 16.1 ppm at 48 h and to 43.9 ± 17.6 ppm at 54 h with very favorable ratios of tumor boron relative to precancerous and normal tissue, making these protocols particularly worthy of radiobiological assessment. Boron concentration values obtained would result in therapeutic BNCT doses in tumor without exceeding radiotolerance in precancerous/normal tissue at the thermal neutron facility at RA-3.

Keywords BNCT · Boron neutron capture therapy · Liposomes · Biodistribution · Experimental oral cancer · Boron

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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 that are taken up preferentially by neoplastic cells to produce selective irradiation of tumor tissue. The high linear energy transfer (LET) alpha particles and recoiling ^7Li nuclei emitted during the ^{10}B (n,α) ^7Li reaction in tissue are known to have a high relative biological effectiveness (RBE) (Coderre and Morris 1999). Their short path length in tissue (6–10 μm) limits their effect

mostly to cells containing ^{10}B atoms, providing a strategy to damage tumor cells while protecting healthy tissue within the treatment volume. The mixed radiation field produced in tissue by BNCT includes the specific boron component (high-LET products of the neutron capture reaction) and the non-specific background dose (gamma photons of the beam plus the interaction of the neutron beam with nitrogen and hydrogen in tissues). BNCT protocols seek to maximize the boron radiation component and minimize the non-selective background dose (Coderre and Morris 1999; Trivillin et al. 2006).

Absolute boron content, distribution, and micro distribution in tumor and healthy tissues are central to the efficacy of BNCT. The requirements for successful BNCT are selective accumulation of a non-toxic ^{10}B carrier in tumor relative to dose-limiting healthy tissues in the treatment volume, a sufficiently high absolute boron concentration of ^{10}B in tumor tissue for sufficient ^{10}B (n,α) ^{7}Li reactions to occur, and targeting of all tumor cell populations to avoid the existence of potentially refractory tumor cells that will impair tumor control (e.g., Coderre and Morris 1999; Heber et al. 2006; Trivillin et al. 2006; Garabalino et al. 2011). In particular, at a given tumor/healthy tissue boron concentration ratio, high absolute ^{10}B tumor concentrations are an asset because they allow for shorter irradiation times and a concomitant reduction in background dose (Coderre and Morris 1999). Furthermore, the microlocalization of ^{10}B also conditions the therapeutic outcome of BNCT (Smith et al. 2001; Santa Cruz and Zamenhof 2004).

Boron biodistribution studies are essential to design and plan useful BNCT preclinical and, ultimately, clinical research protocols. In particular, they identify potentially useful boron compounds and administration protocols and enable the choice of the optimum time post-administration of the boron carrier to perform neutron irradiation, seeking to maximize tumor boron levels while minimizing healthy tissue and blood levels. To date, there is no clinically practical online, noninvasive way to evaluate boron concentration during irradiation for BNCT. Thus, dose calculations are based on boron content values in blood, tumor, and normal tissue obtained from biodistribution studies performed beforehand (e.g., Garabalino et al. 2011). At most, in the case of patient irradiation, blood samples can be taken just before and even during irradiation to infer the tissue boron concentration, assuming the tumor/blood ratios established in previously performed biodistribution studies (González et al. 2004). In the specific case of experimental models, dose calculations are based on the mean values obtained from biodistribution studies in separate sets of animals (Kreimann et al. 2001a). In this sense, it is important to bear in mind that large intra-tumor, inter-tumor, intra-tissue, and inter-subject variations in gross boron content values have been reported (e.g., Heber et al. 2004, 2006). These variations must be accounted for in dose calculation and dose prescription, to avoid exceeding the radiotolerance of the healthy tissues within the treatment volume.

Clinical trials of BNCT for the treatment for glioblastoma multiforme and/or melanoma and, more recently, head and neck tumors and liver metastases, using boronophenylalanine (BPA) or sodium mercaptoundecahydrododecaborane (BSH) as the ^{10}B carriers, have been performed or are underway in Argentina, Europe, Japan, Taiwan, and the United States (e.g., González et al. 2004; Zonta et al. 2006; Kankaanranta et al. 2011a, b; Wang et al. 2011; Nakai et al. 2011; Yamamoto et al. 2011; Lin and Liu 2011). To date, the clinical results have demonstrated the safety and therapeutic potential of this technique. The challenge lies in optimizing BNCT for different pathologies. Adequate experimental models are necessary to examine the potential of different treatment protocols. Contributory translational studies have been carried out employing a variety of experimental models based on the implantation of tumor cells in normal tissue (e.g., Barth et al. 2005). In particular, the optimization of tumor boron delivery has a beneficial effect and is assessed by means of biodistribution studies in experimental models.

To explore new applications and study the radiobiology of BNCT to improve its therapeutic efficacy, we previously proposed and validated the use of the hamster cheek pouch model of oral cancer for BNCT studies (Kreimann et al. 2011a, b). Although progress has been made in the understanding and treatment for head and neck malignancies, their management continues to pose a challenge. Squamous cell carcinoma (SCC) of the head and neck region is the sixth-most common cause of cancer deaths worldwide, and its incidence is rising rapidly in developing countries. The relatively poor overall 5-year survival rate for malignancies of the oral cavity of 58.3 to 63% (Mehrotra et al. 2011) and the fact that radical surgery causes large tissue defect (Kastenbauer and Wollenberg 1999) poses the need for more effective and less toxic therapies that can damage malignant cells selectively, sparing normal cells. The hamster cheek pouch model of carcinogenesis is widely accepted as a model of oral cancer (Salley 1954). Carcinogenesis protocols induce premalignant and malignant changes that closely resemble spontaneous human oral mucosa lesions (Morris 1961). In addition, the hamster cheek pouch model of oral cancer 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. Conversely, the tumor models classically employed in BNCT small-animal studies are based on the growth of implanted cancer cells in healthy tissue (e.g., Barth et al. 2005). In the

hamster cheek pouch, carcinogenesis protocols lead to the development of what has been called, globally, "precancerous tissue" (e.g., Kreimann et al. 2001a) or, more recently, "tissue with potentially malignant disorders (PMD)" (Heber et al. 2010), from which tumors arise. Thus, this mode of tumor induction provides a tumor model surrounded by precancerous tissue. The possibility of studying precancerous tissue in addition to tumor and normal tissue is clinically relevant in terms of its role as a potentially dose-limiting tissue and the fact that second primary tumor locoregional recurrences that arise in field-cancerized tissue are a frequent cause of therapeutic failure (Smith and Haffty 1999; Hoebers et al. 2011).

In previous studies, we demonstrated that potentially therapeutic boron concentrations could be delivered to hamster cheek pouch tumors employing BPA and decahydrodecaborate (GB-10) as the boron delivery agents individually or in combination (e.g., Kreimann et al. 2001a; Heber et al. 2004, 2006). We then demonstrated the therapeutic efficacy of BNCT mediated by BPA and/or GB-10 to treat experimental oral cancer in an experimental model in the hamster cheek pouch with no normal tissue radiotoxicity and without exceeding the radiotolerance of precancerous tissue (Kreimann et al. 2001b; Trivillin et al. 2004, 2006; Heber et al. 2007; Pozzi et al. 2009; Monti Hughes et al. 2009). We also demonstrated the feasibility of treating spontaneous squamous cell carcinomas in felines with BNCT (Rao et al. 2004; Trivillin et al. 2008) and the efficacy of BNCT to inhibit the development of tumors from precancerous tissue (Monti Hughes et al. 2009). More recently, and in light of recent reports by Zonta et al. (2006), we performed boron biodistribution studies in experimental rat models to assess the feasibility of BNCT to treat liver metastasis (Garabalino et al. 2011).

Many of the efforts to improve the efficacy of BNCT have concentrated on the development of novel boronated agents, seeking to maximize absolute tumor boron content and selective uptake. Much attention has been focused on the liposomal delivery system. Liposomes are efficient drug delivery vehicles that are able to deliver large quantities of a wide range of encapsulated agents selectively to tumor tissue. Tumor blood vessels resulting from angiogenesis and vasculogenesis are structurally and functionally abnormal. Blood vessels are leaky, tortuous, and dilated (Jain 1987, 2005) and exhibit fenestrae, vesicles and transcellular holes, widened interendothelial junctions, and a discontinuous or absent basement membrane (Carmeliet and Jain 2000). These aberrant blood vessels allow small liposomes (<100 nm) to pass through, allowing for selective tumor targeting. Furthermore, solid tumors generally exhibit poorly functioning lymphatic drainage. Materials which diffuse into tumorous tissues may persist in the interstitial space for prolonged periods due to the known enhanced permeability and retention (EPR) effect (Maeda and Matsumura 1986). Because liposomes are appropriately sized, they may take advantage of the EPR effect. Therefore, the incorporated agent(s) need not necessarily exhibit tumor affinity. Additionally, the serum half-life of an encapsulated drug is longer than that of the free drug, making it possible to use a lower dose. Because the liposome preserves the structural integrity of the drug, toxicity is often reduced (Li et al. 2006). Several liposomal drugs approved by the US Food and Drug Administration (FDA) are commercially available and are currently being employed in clinical trials (Barenholz 2001).

Small unilamellar liposomes in particular are viewed as potentially useful boron delivery vehicles for BNCT and have been extensively studied by Hawthorne and co-workers (Shelly et al. 1992; Feakes et al. 1994, 1995; Watson-Clarke et al. 1998; Li et al. 2006) and other groups (e.g., Pan et al. 2002; Carlsson et al. 2003; Masunaga et al. 2006; Miyajima et al. 2006; Altieri et al. 2009; Nakamura 2009; Shirakawa et al. 2009; Ueno et al. 2010). They can encapsulate aqueous solutions of sodium salts of polyhedral borane anions and/or incorporate lipophilic boron-containing moieties embedded within the bilayer membrane. The delivery of boron by liposomes incorporating K[nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer membrane and encapsulating a hypertonic buffer (MAC) and by liposomes incorporating K[nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer membrane and encapsulating a concentrated aqueous solution of the hydrophilic species Na_3[ae-$B_{20}H_{17}NH_3$] (MAC-TAC), exhibited potentially therapeutic tumor boron concentration values and tumor selectivity in BALB/c mice bearing EMT6 mammary adenocarcinomas (Feakes et al. 1995).

The aim of the present study was to perform, for the first time, biodistribution studies in a pathology other than mammary adenocarcinomas, i.e., in the hamster cheek pouch oral cancer model employing MAC and MAC-TAC as the boron carriers. In addition, the present study describes normal tissues surrounding tumor, an issue of clinical relevance that has not been previously addressed for these liposomes, seeking to contribute to the optimization of BNCT for the treatment for head and neck cancer by improving boron targeting.

Materials and methods

Tumor induction

Tumors were induced in the right cheek pouch of 44 noninbred young (6 weeks old) Syrian hamsters by topical application of 0.5% of the complete carcinogen dimethyl-1,2-benzanthracene (DMBA) in mineral oil twice a week

for 12 weeks in keeping with a standard hamster cheek pouch carcinogenesis protocol (Shklar et al. 1979) modified as previously described, e.g., (Molinari et al. 2011). The treated pouch was periodically everted under light intraperitoneal (ip) ketamine [70 mg/kg body weight (bw)]-xylazine (10.5 mg/kg bw) anesthesia and examined to monitor tumor development. Once the exophytic tumors, i.e., squamous cell carcinomas, developed and reached a diameter of approximately 3–5 mm, the animals were used for biodistribution studies. This study was conducted in strict compliance with national and institutional guidelines for the care and use of laboratory animals.

Biodistribution studies

Boron compounds

Liposomes were prepared analogously to those described in (Feakes et al. 1995). Briefly, liposomes (volume-weighted mean vesicle diameter $m_v = 61$ nm) incorporating K[nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer and encapsulating a hypertonic PBS buffer (10 mM phosphate/2.7 mM KCl/350 mM NaCl, pH 7.4) (MAC) were prepared with a lipid mixture of distearoylphosphatidylcholine or DSPC/cholesterol/K [nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], 3:3:1. Liposomes ($m_v = 83$ nm) incorporating K [nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] in the bilayer and encapsulating a concentrated (200 mM) aqueous solution of Na_3 [ae-$B_{20}H_{17}NH_3$] (MAC-TAC) were prepared with a lipid mixture of DSPC/cholesterol/K [nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$], 1:1:0.6. The MAC formulation was prepared in two statistically similar batches (868 ± 45 and 904 ± 75 mg B/g), and the MAC-TAC formulation was prepared in a single batch at 1293 ± 71 mg B/g. The liposome suspensions were stored at 4°C, in safelight conditions, for a maximum of 4 months.

Administration protocols

Liposome suspensions were administered as intravenous (iv) bolus injections in the surgically exposed jugular vein of animals (120–170 g bw) anesthetized with an ip injection of ketamine (70 mg/kg bw)—xylazine (10.5 mg/kg bw), followed by skin suture in keeping with a technique developed previously (Schwint et al. 1984). If the injection volume exceeded 1 ml, 2 sequential injections were given 5 min apart because volumes in excess of 1.5 ml administered as a single iv injection are poorly tolerated by the animals. MAC was administered at a dose of 6 mg B/kg bw (approximately 0.69 ml/100 g bw), and MAC-TAC was administered at a dose of 18 mg B/kg bw (approximately 1.39 ml/100 g bw). In view of the fact that MAC-TAC proved to be the compound with the best therapeutic potential (see "Results" section), an additional group of 3 tumor-bearing hamsters were injected with MAC-TAC and followed for 28 days [the traditional follow-up period employed in tumor control studies in the hamster cheek pouch oral cancer model (e.g., Kreimann et al. 2001b; Trivillin et al. 2006; Pozzi et al. 2009; Molinari et al. 2011)] to assess potential signs of toxicity in terms of clinical status and body weight.

Blood and tissue sampling

Blood and tissue samples were taken 16, 30, and 48 h after administration of MAC and 16, 30, 48, 54, and 72 h after administration of MAC-TAC. These times were selected based on a previous study in experimental mammary adenocarcinoma in BALB/c mice (Feakes et al. 1995) and are considerably longer than the 3–4 h intervals characteristically used for low molecular weight, non-encapsulated boron carriers such as BPA and GB-10. As mentioned above, in the case of liposomes, transport out of the circulation into tumor tissue is favored by the leaky tumor neovasculature (Watson-Clark et al. 1998) and would take longer than the diffusion out of the circulation of free, low molecular weight boron compounds (Barth et al. 2005). The known enhanced permeability and retention (EPR) effect in tumors (Maeda and Matsumura 1986) and the longer circulation life span of an encapsulated drug would allow for the slow build-up of tumor boron concentration in the case of liposomes. The fact that encapsulated drugs circulate for longer periods of time, are delivered more slowly and are retained longer than free drugs explains the choice of longer post-administration times. Five to six animals were evaluated per group. Blood samples were taken from the surgically exposed jugular vein under ketamine (140 mg/kg bw)—xylazine (21 mg/kg bw) anesthesia. The animals were then killed by overdose of anesthesia immediately prior to tissue sampling. Samples of tumor, precancerous tissue, normal pouch tissue, spleen, liver, and kidney were taken for each animal.

Boron analysis

All of the samples were weighed immediately. Until use, tissue samples were stored at −20°C and blood samples were stored with EDTA 5% v/v at 4°C. The samples were processed for gross boron measurement by inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC2, Perkin Elmer). Tissue samples (approximately 50 mg) and blood samples (200–300 μl) were digested in 15 ml Falcon tubes for 1 h at 100°C in 0.25 ml of a 1:1 mixture of ultrapure concentrated sulfuric acid (J.T. Baker, Phillipsburg, USA) and sub-boiling nitric acid distilled from nitric acid 65% (p.a., Carlo Erba, Milan, Italy). Once the digestion process was complete, the mixture was

allowed to cool and milli-Q water was added to bring the final volume to 10 ml. The digested samples were stored at room temperature for a maximum of 7 days prior to measurement. All the digested samples were vortexed immediately prior to preparation for actual measurement. Approximately 0.5–1 ml of the digested tissue sample or 0.2 ml of the digested blood sample (depending on estimated boron content) was placed in a new Falcon tube and mixed with 0.20 ml of a 1:1 mixture of ultrapure concentrated sulfuric acid and sub-boiling nitric acid. About 0.25 ml of ^{6}Li (1 ppm) was added as an internal standard. Milli-Q water was added to bring the final volume to 10 ml. All the prepared samples were vortexed immediately prior to measurement. Different dilutions of a standard calibration solution (Multi-Element ICP-MS Calibration Standard of B, Ge, Mo, Nb, P, Re, S, Si, Ta, Ti, W, Zr, 10 mg/l, Perkin Elmer) were used to prepare a calibration line each day of operation.

End points

Absolute boron concentrations in tumor, blood, and clinically relevant normal tissues were evaluated for each of the compounds and post-administration time-points. Tumor/blood and tumor/normal pouch tissue boron concentration ratios were calculated for each of the tumors. Each hamster had a variable number of tumors, and the number of subsamples measured per tumor depended on tumor size. Ratios were calculated for each tumor considering the mean value of the subsamples corresponding to that particular tumor and the mean normal pouch tissue or blood value corresponding to the hamster bearing that particular tumor. The tabulated ratio values correspond to the mean value ± standard deviation of the ratio for each of the tumors.

Results

No ostensible signs of toxicity were observed with the selected formulations of MAC and MAC-TAC liposomes.

Based on previous BNCT radiobiological studies in the hamster cheek pouch oral cancer model with other boron compounds (e.g., Kreimann et al. 2001b; Trivillin et al. 2006; Molinari et al. 2011), we previously defined the following guidelines to establish the potential therapeutic value of the boron carriers, the administration protocols, and time-points post-administration (Garabalino et al. 2011):

- No manifest toxicity
- Absolute boron concentration in tumor >20 ppm
- Boron concentration ratio tumor/normal tissue >1
- Boron concentration ratio tumor/blood >1

Although the actual usefulness of a particular boron carrier and protocol can only be determined by in vivo radiobiological BNCT studies, our previous studies in the hamster cheek pouch oral cancer model with other boron compounds suggest that the protocols that meet the above requirements are potentially therapeutic and warrant radiobiological assessment.

The time-course biodistribution of MAC is shown in Table 1. Although tumor uptake versus normal pouch tissue was remarkably selective with ratios >12:1, absolute boron concentration in tumor was suboptimal, with values ranging from approximately 15–18 ppm. Little or no selectivity was observed for tumor as compared to blood values. However, the data suggest an improvement in tumor/blood ratios at the later time-points. No accumulation was observed in spleen, liver, or kidney.

The time-course biodistribution of MAC-TAC is presented in Table 2 and Fig. 1. High absolute tumor boron concentration values ranging from approximately 40–70 ppm were observed for 16, 30, 48, and 54 h. At 72 h post-administration, tumor values fell considerably to approximately 20 ppm. Tumor uptake versus normal pouch tissue was markedly selective, with ratios ranging approximately from 8:1 to 27:1. Normal pouch tissue absolute boron values ranged from approximately 2–8 ppm. Precancerous tissue values ranged from approximately 4–15 ppm, somewhat above normal pouch tissue values. Tumor/blood ratios were lower than the tumor/normal pouch tissue ratios but showed tumor selectivity, particularly at 48, 54, and 72 h when ratios ranged approximately from 1.9:1 to 3:1 (Table 2). Although tumor selectivity was higher at 72 h, absolute tumor boron values barely reached the 20 ppm threshold established for therapeutic usefulness. Conversely, although tumor boron values were highest in tumor at 16 h, concurring high blood boron values might be a concern in terms of radiotoxicity. Within this context, the 48 and 54 h time-points would hold the highest therapeutic potential (Fig. 2). No accumulation was observed in liver or kidney at these time-points. Moderate accumulation was seen in spleen at 48 h. However, assuming spleen levels of the compound are not chemically toxic in themselves, moderately high spleen levels would not be a concern for head and neck cancer, where the spleen would not be in the treatment volume. In particular, in the case of the hamster cheek pouch oral cancer model, the body of the animal is shielded while the tumor-bearing pouch is exposed to the neutron beam as previously described (e.g., Molinari et al. 2011).

As previously described for other boron compounds in different experimental models and in different tumor types in patients (e.g., Heber et al. 2006; Cardoso et al. 2009), the spread in tumor boron values was remarkably large.

Table 1 Boron concentration (mean ± standard deviation) (ppm) in blood and tissue samples for the MAC liposomes at different times post-administration as indicated; *n* denotes number of animals or tumors; each hamster had a variable number of tumors

Tissue/time	16 (h)	30 (h)	48 (h)
Blood	29.1 ± 8.8	17.7 ± 7.7	14.5 ± 5.1
	$n = 5$	$n = 6$	$n = 5$
Tumor	15.2 ± 7.5	14.6 ± 3.0	18.4 ± 4.1
	$n = 14$	$n = 16$	$n = 10$
Precancerous pouch tissue	5.8 ± 2.9	6.9 ± 3.1	4.8 ± 2.0
	$n = 5$	$n = 6$	$n = 5$
Normal pouch tissue	1.7 ± 0.9	1.4 ± 0.6	1.4 ± 0.5
	$n = 5$	$n = 6$	$n = 5$
Spleen	14.9 ± 8.5	12.6 ± 6.2	10.6 ± 2.1
	$n = 5$	$n = 6$	$n = 5$
Liver	10.8 ± 4.8	6.6 ± 2.0	4.1 ± 0.7
	$n = 5$	$n = 6$	$n = 5$
Kidney	8.4 ± 2.4	4.8 ± 0.7	4.7 ± 1
	$n = 5$	$n = 6$	$n = 5$
Tumor/Blood	0.6 ± 0.1	0.9 ± 0.3	1.3 ± 0.6
	$n = 5$	$n = 5$	$n = 5$
Tumor/Normal pouch tissue	14.6 ± 11.1	12.2 ± 5.8	14 ± 7.1
	$n = 5$	$n = 5$	$n = 5$

The number of samples measured per tumor depended on tumor size. Tumor/blood and tumor/normal pouch tissue ratios were calculated for each tumor considering the mean value of the samples corresponding to that particular tumor and the mean blood or normal pouch tissue value corresponding to the animal bearing that particular tumor. The tabulated ratios correspond to the mean ± standard deviation of the ratios for each of the tumors

Table 2 Boron concentration (mean ± standard deviation) (ppm) in blood and tissue samples for the MAC-TAC liposomes at different times post-administration as indicated; *n* denotes number of animals or tumors; each hamster had a variable number of tumors

Tissue/time	16 (h)	30 (h)	48 (h)	54 (h)	72 (h)
Blood	122.2 ± 30.8	49.6 ± 18.4	34.1 ± 5.9	18.2 ± 8.2	6.7 ± 1.7
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Tumor	71.5 ± 34.8	48.4 ± 19.1	66.6 ± 16.1	43.9 ± 17.6	20.1 ± 10.8
	$n = 18$	$n = 12$	$n = 11$	$n = 10$	$n = 17$
Precancerous tissue	15.1 ± 4.3	11.7 ± 6.0	11.3 ± 6.2	9.6 ± 2.2	4.5 ± 2.4
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Normal pouch tissue	8.1 ± 1.2	6.9 ± 6.1	7.0 ± 5.5	1.9 ± 1.2	2.2 ± 2.2
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Spleen	106.6 ± 39.2	73.6 ± 55.2	69.3 ± 10.6	26.1 ± 7.9	15.0 ± 7.7
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Liver	81.9 ± 20.9	37.6 ± 9.7	31.3 ± 7.5	19.7 ± 1.8	16.7 ± 7.5
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Kidney	30.0 ± 4.9	24.5 ± 1.6	23.1 ± 1.9	16.3 ± 3.8	10.6 ± 4.2
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Tumor/blood	0.5 ± 0.1	1.1 ± 0.4	1.9 ± 0.5	2.8 ± 2.1	3.0 ± 0.9
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$
Tumor/normal pouch tissue	8.8 ± 3.4	12.4 ± 8.1	12.9 ± 6.8	27.7 ± 10.9	26.8 ± 34.9
	$n = 5$	$n = 5$	$n = 5$	$n = 5$	$n = 5$

The number of samples measured per tumor depended on tumor size. Tumor/blood and tumor/normal pouch tissue ratios were calculated for each tumor considering the mean value of the samples corresponding to that particular tumor and the mean blood or normal pouch tissue value corresponding to the animal bearing that particular tumor. The tabulated ratios correspond to the mean ± standard deviation of the ratios for each of the tumors

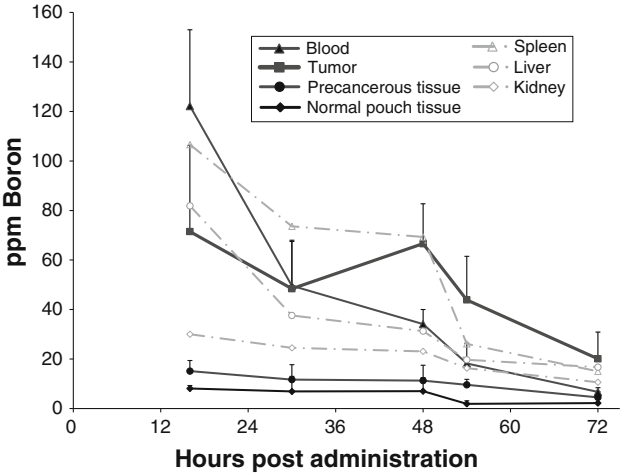

Fig. 1 Time-course biodistribution of MAC-TAC liposomes: boron concentration (mean + SD) for each of the tissues; For the sake of clarity, the SD bars were only included for the most relevant tissues. All SD values are presented in Table 2

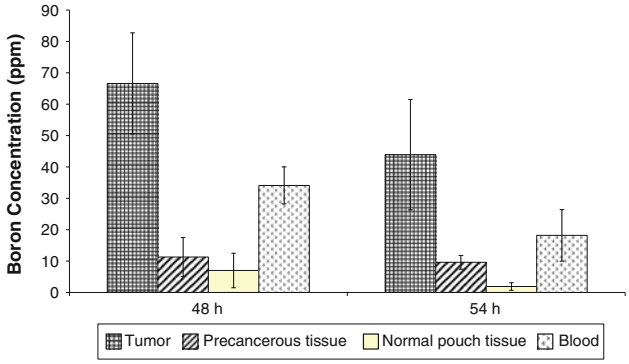

Fig. 2 Boron concentration values (mean ± SD) for MAC-TAC liposomes in the clinically most relevant tissues at 48 and 54 h post-administration, the time-points with greatest therapeutic potential

Discussion

In the present study, for the first time, the time-course biodistribution of boron delivered by MAC and MAC-TAC liposomes in the hamster cheek pouch oral cancer model is reported. It was possible to selectively deliver potentially therapeutic amounts of boron to hamster cheek pouch tumor by iv administration of MAC-TAC and achieve ratios between tumor and normal pouch tissue and tumor and blood that would be compatible with treatment. The biodistribution data obtained in the present study indicate potentially therapeutic absolute and relative boron concentration values particularly during the 48–54 h post-administration period. Although the normal tissue and blood boron values at 72 h were lower than at the earlier time-points and thus conceivably more advantageous, the concurrent absolute boron content in tumor of approximately 20 ppm at 72 h would be only marginally useful. Conversely, high tumor boron values at 16 and 30 h were associated with high blood values that might pose a concern in terms of potential radiotoxicity. MAC delivered boron selectively to tumor, but absolute boron content was suboptimal. The potential benefits and toxicity of administering a higher dose of MAC warrant evaluation. Although MAC employed as a stand-alone boron carrier at the dose-level selected based on previous studies (Feakes et al. 1995) delivered what appear to be insufficient amounts of boron to tumor, its use in combination with another boron compound might provide a therapeutic advantage. Because targeting of all tumor populations within a heterogeneous tumor is critical to the success of BNCT, it has been postulated that the combined administration of different boron compounds with different properties and complementary uptake mechanisms may enhance the therapeutic efficacy of BNCT (Ono et al. 1999; Trivillin et al. 2006; Heber et al. 2006).

Of particular concern in oral cancer is the boron content in precancerous tissue. As previously established, precancerous tissue is the dose-limiting tissue in the hamster cheek pouch oral cancer model. Dose escalation is limited by mucositis in this tissue (e.g., Molinari et al. 2011). In a clinical scenario, confluent oral mucositis is a frequent, dose-limiting side effect during conventional radiotherapy for advanced head and neck tumors (Coderre and Morris 1999; Sonis et al. 2004). Within this context, the low boron content delivered by MAC and MAC-TAC to precancerous tissue (Tables 1, 2; Fig. 1), ranging from approximately 4–15 ppm, would be an asset. Additionally, the fact that precancerous tissue boron values are, overall, higher than normal pouch tissue values, would make it potentially possible to achieve a therapeutic effect in precancerous tissue in terms of inhibition of tumor development without significant damage to normal pouch tissue (Heber et al. 2007; Monti Hughes et al. 2009).

Admittedly, the implications of the observed gross boron content values in terms of biological effect can only be determined with in vivo BNCT radiobiological studies. However, the therapeutic potential of the different administration protocols and boron compounds described here is suggested based on the biodistribution data and previous radiobiological studies in the hamster cheek pouch oral cancer model employing other boron carriers (e.g., Kreimann et al. 2001b; Trivillin et al. 2004; 2006; Pozzi et al. 2009, Monti Hughes et al. 2009; Molinari et al. 2011). It is known that the biological effect of BNCT depends on the relative biological effectiveness factors (RBE) of the high-LET and low-LET dose components of BNCT. Boron micro distribution phenomena determine the RBE factor for each boron carrier in a particular tissue, referred to as the compound biological effectiveness factor (CBE)

(Morris et al. 1994) of a particular boron carrier in a particular tissue. In this way, biodistribution studies serve as a guideline to establish the boron carriers and protocols that would be worthy of radiobiological evaluation.

The incorporation of both K [nido-7-$CH_3(CH_2)_{15}$-7,8-$C_2B_9H_{11}$] and the hydrophilic species Na_3 [ae-$B_{20}H_{17}NH_3$] within the same liposomes improved maximum tumor boron concentrations. MAC-TAC would pose an advantage in terms of absolute tumor boron content over other boron compounds such as BPA and GB-10 that have been used as single boron carriers (Kreimann et al. 2001a, Heber et al. 2004) in the hamster cheek pouch oral cancer model. Maximum tumor boron values achieved with MAC-TAC are approximately 20–30% higher than those achieved with BPA or GB-10 administration protocols that in turn resulted in overall successful tumor response rates (partial response + complete response) of 70–90% (e.g., Kreimann et al. 2001b, Trivillin et al. 2006). In addition, MAC-TAC tumor/normal pouch tissue selectivity was greater than that reported for BPA and GB-10 in this model (Kreimann et al. 2001a; Heber et al. 2004), conceivably allowing for an improved therapeutic ratio between tumor and healthy tissues. Tumor retention times are considerably longer for MAC-TAC than for BPA and GB-10. This makes it possible for tumor to maintain therapeutic values over a 48–54 h period (compared to 3–4 h for BPA and GB-10), while providing an opportunity for the boron concentrations in other tissues, particularly blood, to decrease. This property is attributed to the susceptibility of Na_3 [ae-$B_{20}H_{17}NH_3$] to undergo intracellular oxidation followed by nucleophilic attack and reaction with intracellular protein moieties (Feakes et al. 1994). Although high absolute tumor boron values, selective tumor uptake, and long retention times are all potential assets, as previously stated, it cannot be stressed enough that actual radiobiological efficacy remains to be determined with in vivo studies. Regarding the spread in absolute tumor boron values observed herein, the high variability and heterogeneity of boron concentrations in tumor tissue are a general issue of concern in BNCT (e.g., Ono et al. 1999; Trivillin et al. 2006). Even multiple tumor samples from the same patient can exhibit considerable variation in boron concentration delivered by BPA. This heterogeneity would be largely due to features such as biological diversity between pathological cells and varying blood flow within tumor (Coderre et al. 1998; Gibson et al. 2003). Tumor/normal tissue boron concentration ratios for human squamous cell carcinoma treated with BNCT mediated by BPA in a clinical trial ranged from 1.8 to 4.4 (Kato et al. 2004). Within this context, the high absolute tumor boron content and tumor/normal pouch tissue ratios achieved with MAC-TAC in the present study are particularly useful because even the lowest value of the wide range would be potentially useful for BNCT.

Dose calculations were performed employing the boron values corresponding to the protocols with greatest therapeutic potential (MAC-TAC, 48 and 54 h post-administration) to examine the feasibility of performing in vivo BNCT studies at the previously characterized RA-3 thermal neutron facility (Miller et al. 2009; Pozzi et al. 2009). These calculations took into account the use of an enclosure built of lithium carbonate (enriched to 95% in ^{6}Li) to shield the body of the animal while everting the tumor-bearing pouch out of the enclosure onto a protruding shelf. The thermal neutron flux is about 8.2×10^9 n cm^{-2} s^{-1} in the outermost position on the pouch shelf and 7×10^9 n cm^{-2} s^{-1} in the center position. These values are approximately 25% lower than the unperturbed flux at this location, largely due to local flux depression by the shield enclosure. The thermal neutron flux at all locations within the shield container is at least a factor of 20 lower than the flux on the pouch shelf. The dose rate of gamma rays in air at the irradiation location is 6.5 ± 0.5 Gy h^{-1}. Based on these values, MAC-TAC (48 and 54 h post-administration) could deliver 7.5 Gy total physical absorbed dose (uncorrected for RBE and CBE values) to tumor in an exposure time range of 3–4.5 min. The associated total physical absorbed dose to exposed normal pouch tissue would be approximately 0.6–1.9 Gy, whereas the associated total physical absorbed dose to the dose-limiting precancerous tissue surrounding tumors in an exposed tumor-bearing pouch would be approximately 1.5–2.6 Gy. Based on previous BNCT studies in the hamster cheek pouch oral cancer model employing different boron carriers (Pozzi et al. 2009; Monti Hughes et al. 2009; Molinari et al. 2011), these physical dose ranges are in keeping with therapeutically useful doses in terms of tumor response and toxicity. In particular, the physical absorbed dose to the dose-limiting precancerous tissue is well below the maximum physical absorbed dose of 5 Gy that is routinely prescribed to precancerous tissue in this model when BPA is used as the boron carrier. Furthermore, in the case of BNCT mediated by GB-10, precancerous tissue tolerates total physical absorbed doses of up to approximately 8 Gy. In a BNCT clinical trial for recurred head and neck cancer, the mucosal membrane absorbed physical dose was selected as the dose-limiting factor and limited to 6 Gy or less for each of the two BNCT treatments administered (Kankaanranta et al. 2011b). In this sense, tumor dose escalation from the suggested 7.5 Gy could be envisioned without exceeding the tolerance of dose-limiting precancerous tissue.

The results of the present study suggest the therapeutic potential of boron-bearing liposomes in terms of gross boron biodistribution values. In particular, MAC-TAC administered iv at a dose of 18 mg B/kg bw would be particularly attractive to examine in neutron irradiation

studies at 48 and 54 h post-administration. Finally, it is important to note that boron microlocalization and targeting homogeneity are also pivotal to tissue response but cannot be quantified solely on the basis of gross boron determinations. However, neutron irradiation studies would provide some indirect information that would in fact aid in the understanding of these issues along with providing direct macroscopic tissue response data.

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