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ORIGINAL ARTICLE

Boron neutron capture therapy for oral precancer: proof of principle in an experimental animal model

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OBJECTIVES: Field-cancerized tissue can give rise to second primary tumours, causing therapeutic failure. Boron neutron capture therapy (BNCT) is based on biological targeting and would serve to treat undetectable foci of malignant transformation. The aim of this study was to optimize BNCT for the integral treatment for oral cancer, with particular emphasis on the inhibitory effect on tumour development originating in precancerous conditions, and radiotoxicity of different BNCT protocols in a hamster cheek pouch oral precancer model.

MATERIALS AND METHODS: Groups of cancerized hamsters were locally exposed to *single* or *double* (2 or 4 weeks apart) applications of BNCT at different dose levels, mediated by the boron compounds boronophenylalanine (BPA) or BPA and decahydrodecaborate (GB-10) administered jointly. Cancerized, sham-irradiated hamsters served as controls. Clinical status, tumour development from field-cancerized tissue and mucositis were followed for 8 months.

RESULTS: A *double* application (4 weeks apart) of BNCT mediated by GB-10+ BPA at a total dose of 10 Gy in two 5-Gy doses rendered the best therapeutic advantage (63–100% inhibition of tumour development from field-cancerized tissue), minimizing dose-limiting mucositis.

CONCLUSION: BNCT can be optimized for the integral treatment for head and neck cancer, considering the implications for field-cancerized tissue.

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Keywords: boron neutron capture therapy; hamster cheek pouch oral precancer model; field cancerization; precancerous conditions; oral cancer

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Introduction

Boron neutron capture therapy (BNCT) is a binary treatment that combines the administration of boron carriers that are taken up preferentially by neoplastic tissue and irradiation with a thermal/epithermal neutron beam. The high-linear energy transfer (LET) α particles and recoiling ^{7}Li nuclei emitted during the capture of a thermal neutron by a ^{10}B nucleus have a high relative biological effectiveness. Their short range in tissue (6–10 μm) would limit the damage largely to cells containing ^{10}B. In this way, BNCT would target neoplastic tissue selectively, sparing normal tissue. However, the interaction of the neutrons with nitrogen and hydrogen in tissue and the gamma component of the beam will deliver an unavoidable and non-specific background dose (Coderre and Morris, 1999; Trivillin *et al*, 2006). As BNCT is based on biological rather than geometric targeting, it would be suited to treat undetectable micrometastases (Cardoso *et al*, 2007) and foci of malignant transformation in field-cancerized tissue (Monti Hughes *et al*, 2009, 2011).

Clinical studies of BNCT for glioblastoma multiforme and/or melanoma and, more recently, head and neck tumours and liver metastases have been performed or are underway in the United States, Japan, Europe, Argentina and Taiwan (e.g. Chanana *et al*, 1999; Gonzalez *et al*, 2004; Zonta *et al*, 2006; Suzuki *et al*, 2007; Kankaanranta *et al*, 2011, 2012; Wang *et al*, 2011; Barth *et al*, 2012). To date, the clinical results have shown a potential therapeutic advantage, with room for improvement.

The relatively poor overall 5-year survival rate for malignancies of the oral cavity (Mehrotra *et al*, 2011) poses the need for more effective and selective therapies. Studies in appropriate experimental models are pivotal to progress in this field.

The hamster cheek pouch model of oral cancer was previously proposed by our group for BNCT studies (Kreimann *et al*, 2001a,b). Our first experimental studies preceded the first clinical trial of BNCT for head and neck malignancies (Kato *et al*, 2004). The hamster cheek pouch

model of carcinogenesis is widely accepted as a model of oral cancer (Kreimann *et al*, 2001a) and oral mucositis (Bowen *et al*, 2011). Carcinogenesis protocols induce premalignant and malignant changes that closely resemble spontaneous human oral mucosa lesions (Kreimann *et al*, 2001a). We previously demonstrated the therapeutic efficacy of BNCT mediated by the boron compounds boronophenylalanine (BPA) and/or decahydrodecaborate (GB-10) to treat oral cancer in this experimental model with no normal tissue radiotoxicity, and slight/moderate mucositis in dose-limiting precancerous tissue around tumours (Kreimann *et al*, 2001a).

Despite the success of the BNCT protocols employed in these studies to treat tumours, the inhibition of tumour development from field-cancerized tissue remains an unresolved challenge. Different terms have been used to refer to oral mucosa that can give rise to the development of multiple oral tumours, that is, precancerous tissue (Kreimann *et al*, 2001a), premalignant fields (Gonzalez-Moles *et al*, 2012), tissue with potentially malignant disorders (PMD) (Heber *et al*, 2010; Sarode *et al*, 2012), precancerous condition (Sarode *et al*, 2012) and field-cancerized tissue (Braakhuis *et al*, 2003). The term 'field-cancerized tissue' or 'precancerous condition' will be used henceforth to describe a tissue with a significantly increased risk of cancer.

The relevance of field cancerization in head and neck cancer lies in the frequent occurrence of second primary tumours after treatment (Ge *et al*, 2010). There is a risk of approximately 20% for second primary tumours based on continued exposure to risk factors. In addition, in head and neck cancer, the incidence of recurrent disease may be as high as 30–50% after radiotherapy (e.g. Hoebers *et al*, 2011). Within this context, recurrent and/or second primary tumours are a therapeutic challenge in head and neck cancer. In addition, the constraints imposed on therapeutic protocols by the dose-limiting nature of field-cancerized tissue must be assessed. In a clinical scenario, confluent oral mucositis is a frequent, dose-limiting side effect during conventional radiotherapy for advanced head and neck tumours (Sonis, 2004).

Within this context, the hamster cheek pouch oral cancer model poses a unique advantage in that it allows for the study of both tumours and field-cancerized tissue (Braakhuis *et al*, 2003; Heber *et al*, 2007). However, the aggressiveness of the model as employed in tumour control studies (e.g. Kreimann *et al*, 2001b; Trivillin *et al*, 2006; Pozzi *et al*, 2009; Molinari *et al*, 2011, 2012) precludes the *long-term* follow-up needed to evaluate the effect of BNCT on field-cancerized tissue in terms of the development of recurrent and/or second primary tumours (Chen *et al*, 2011). Thus, we developed a model of oral precancer or field-cancerized tissue in the hamster cheek pouch that allows for *long-term* studies, that is, is amenable to *long-term* follow-up but, left untreated, guarantees tumour development in $\geq 90\%$ of the animals (Heber *et al*, 2010). Being less aggressive, it mimics oral carcinogenesis more closely (Morris *et al*, 2011). Employing this model, we demonstrated the partial inhibitory effect on the development of tumours of a *single* application of BNCT mediated by BPA, GB-10 or (GB-10+ BPA) at 4 Gy absorbed dose prescribed to field-cancerized tissue, with no normal tissue radiotoxicity and without severe mucositis in dose-limiting field-cancerized tissue (Monti Hughes *et al*, 2009). We then demonstrated that a *double* application of BPA-BNCT and (GB-10+ BPA)-BNCT at 8 Gy total absorbed dose in two 4-Gy doses administered 6 weeks apart could be used therapeutically at no additional cost in terms of radiotoxicity (Monti Hughes *et al*, 2011).

Seeking to optimize BNCT in terms of improving therapeutic efficacy and reducing radiotoxicity, the aim of the present study was to contribute to the knowledge of BNCT radiobiology for oral precancer and assess new BNCT protocols in terms of inhibition of tumour development and radiotoxicity in the hamster cheek pouch model of oral precancer for *long-term* studies.

Materials and methods

Model of oral precancer for long-term studies

Six-week-old Syrian hamsters were treated by topical application of 0.5% dimethylbenzanthracene in mineral oil in the right cheek pouch, twice a week for 6 weeks (Heber *et al*, 2010), and then assigned to the control group (cancerized, sham-irradiated, i.e. matched manipulation, no treatment) and different experimental groups for radiobiological BNCT studies as indicated. Studies were initiated 1 week after the completion of the carcinogenesis protocol (T0). As previously described, the histological analysis of field-cancerized tissue induced by the 6-week protocol confirmed the existence of the same histological categories that are known to exist in the tissue with PMD induced by the classical 12-week protocol, that is, NUMF (no unusual microscopic features): an epithelium with no apparent lesions, but with subepithelial fibrosis; hyperplasia; dysplasia. These areas coexist with tumours (Heber *et al*, 2007, 2010). Experiments were carried out in accordance with the guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures and in accordance with local laws and regulations. Adequate measures were taken to minimize pain or discomfort.

Radiobiological BNCT studies

Cancerized animals were assigned to the following experimental groups and treated as indicated:

1. *D*BNCT (2 weeks apart, 8 Gy): *Double* application of BNCT at 8 Gy total absorbed dose prescribed to field-cancerized tissue, in two 4-Gy doses administered 2 weeks apart [interval chosen based on tissue response in our previous studies (Monti Hughes *et al*, 2009, 2011)]: *D*BPA-BNCT ($n = 11$); *D*(GB-10+ BPA)-BNCT ($n = 10$); beam only (*D*BO) (equal neutron fluence to match the longest exposure time corresponding to the BPA-BNCT group) to assess the effect of background dose ($n = 10$). Normal (non-cancerized) counterparts were treated with each of the protocols to assess normal pouch tissue response ($n = 6$ for each protocol).
2. *S*BNCT (8 Gy): *Single* application of BNCT at 8 Gy absorbed dose: *S*BPA-BNCT ($n = 6$); *S*(GB-10+

BPA)-BNCT ($n = 6$); *S*BO ($n = 6$). Normal counterparts were treated with *S*BPA-BNCT ($n = 6$) (the protocol known to induce severest mucositis).

Based on tissue response in these groups and a computational modelling study (Farías *et al*, 2011) to explore dose inhomogeneities within the hamster pouch as the potential cause of variations in tissue response (see Results), the following protocols were tested:

1. *S*BPA-BNCT (6 Gy): *Single* application of BPA-BNCT at 6 Gy absorbed dose ($n = 4$).
2. *D*BNCT (10 Gy, 4 weeks apart): *Double* application of BNCT at 10 Gy total absorbed dose in two 5-Gy doses administered 4 weeks apart (the interval was chosen based on tissue response in our previous studies by Monti Hughes *et al*, 2009, 2011 and this study [see Results]): *D*BPA-BNCT ($n = 6$); *D*(GB-10+ BPA)-BNCT ($n = 6$); *D*BO ($n = 6$). Normal counterparts were treated with *D*BPA-BNCT ($n = 4$).

In the case of BNCT protocols involving the administration of BPA alone, BPA was administered as a bolus intraperitoneal (ip) injection at a dose of 15.5 mg ^{10}B/kg. Neutron irradiation was performed 3 h postadministration of BPA. In the case of the BNCT protocols involving the combined administration of GB-10 and BPA, BPA was administered as nine ip injections at a total dose of 31 mg ^{10}B/kg over 1.5 h, and GB-10 was administered as a bolus intravenous (iv) injection in the jugular vein at a dose of 34.5 mg ^{10}B/kg. It must be noted that attempts to increase the bolus injection volume were poorly tolerated by the animals. Furthermore, BPA was administered as fractionated ip injections to simulate an infusion (Garabalino *et al*, 2011). Neutron irradiation was performed 3 h postadministration of GB-10 and 1.5 h postadministration of the last injection of BPA. Dose calculations were based on previously reported boron biodistribution data in this model (Monti Hughes *et al*, 2009, 2011).

The control group consisted of 88 cancerized, sham-irradiated (matched manipulation, no treatment) hamsters. All available controls were pooled.

The animals were irradiated at the different dose levels (Table 1) at the RA-3 thermal facility employing a lithium-6 carbonate shielding to protect the body of the animal while the cheek pouch is everted out of the enclosure onto a protruding shelf for exposure (Monti Hughes *et al*, 2011). The mean thermal neutron flux at the centre of the shelf was $7.49 \times 10^9 \pm 1.6 \times 10^9$ n cm^{-2} s^{-1}, and the mean gamma dose rate at the irradiation position was 6.08 ± 0.61 Gy h^{-1}.

Follow-up

The clinical signs of the animals were monitored regularly. Potential development of recurrent and/or second primary tumours (defined as tumours that were not present at the time of the first irradiation) from field-cancerized tissue was assessed weekly by visual inspection for 8 months after the first irradiation (T0).

At the same time points, potential radiotoxicity in terms of mucositis was monitored in field-cancerized tissue and in normal pouch tissue. The severity of mucositis was evaluated semiquantitatively according to an adaptation of oral mucositis scales (Sonis *et al*, 2000; López Castaño *et al*, 2005), that is, Grade 0: healthy appearance, no erosion or vasodilation; Grade 1 (slight): erythema and/or oedema and/or vasodilation, no evidence of mucosal erosion; Grade 2 (slight): severe erythema and/or oedema, vasodilation and/or superficial erosion; Grade 3 (moderate): severe erythema and/or oedema, vasodilation and formation of ulcers <2 mm in diameter; Grade 4: severe erythema and/or oedema, vasodilation and formation of ulcers ≥ 2 mm and <4 mm in diameter and/or areas of necrosis <4 mm in diameter; Grade 5 (severe): formation of ulcers ≥ 4 mm in diameter or multiple ulcers ≥ 2 mm in diameter and/or areas of necrosis ≥ 4 mm in diameter.

Statistical analysis

When pertinent, statistical analysis of the data was performed using Fisher's exact test. Statistical significance was set at $P = 0.05$.

Results

For all BNCT protocols (*D*BNCT and *S*BNCT) and BO protocols (*D*BO and *S*BO), the animals did not exhibit any clinical signs of radiotoxicity throughout the follow-up period of 8 months. Seeking to improve the inhibitory effect of BNCT on tumour development from field-cancerized tissue reported by Monti Hughes *et al* (2011), we first shortened the interval between irradiations from 6 to 2 weeks to conceivably reduce repopulation without increasing mucositis, still prescribing 4 Gy absorbed dose to field-cancerized tissue for each irradiation (8 Gy total

Table 1 Total absorbed doses (Gy) for the different BNCT treatments. For *D*BNCT experiments, data are quoted for each of two similar irradiations

Treatment	*Protocol*	*Total absorbed doses (Gy) Field-cancerized tissue*
Double application (8 Gy in two 4-Gy doses administered 2 weeks apart)	*D*BO	0.90 ± 0.10
	*D*BPA-BNCT	4.0 ± 1.5
	D(GB-10+ BPA)-BNCT	4.1 ± 1.1
Single application (8 Gy)	*S*BO	2.3 ± 0.22
	*S*BPA-BNCT	8.0 ± 2.4
	S(GB-10+ BPA)-BNCT	8.0 ± 2.2
Single application (6 Gy)	*S*BPA-BNCT	6.0 ± 1.2
Double application (10 Gy in two 5-Gy doses administered 4 weeks apart)	*D*BO	1.17 ± 0.33
	*D*BPA-BNCT	4.6 ± 1.6
	D(GB-10+ BPA)-BNCT	5.0 ± 2.1

dose). T50 and T80, that is, the times at which 50% and 80% of the animals, respectively, exhibited recurrent and/or second primary tumours, are shown in Table 2. T50 provided evidence of a moderate inhibitory effect on tumour development of all the protocols *vs* the control group. T80 revealed a slight inhibitory effect for *D*BPA-BNCT and a marked inhibitory effect of *D*(GB-10+ BPA)-BNCT. The therapeutic efficacy of the beam-only protocol was unexpected and counterintuitive.

Mucositis in field-cancerized tissue was slight/moderate (Grades 1–3), resolving (reversion to G0/G1) by the 4th week after the second irradiation. Mucositis after the second application was not exacerbated by the first application. Comparing the *D*BNCT protocol with a 6-week interval (Monti Hughes *et al*, 2011) with the *D*BNCT with a 2-week interval (this study), shortening the interval between applications from 6 to 2 weeks did not improve the therapeutic efficacy of the BNCT treatments in terms of inhibition of tumour development, except in the case of the *long-term* effect of *D*(GB-10+ BPA)-BNCT (Table 2). Shortening the interval between irradiations did not cause severe mucositis. The *D*BNCT protocols induced only G0-G2 mucositis in normal tissue.

Due to the overall lack of improvement in therapeutic effect of this new *D*BNCT protocol (*D*BNCT at 8 Gy, in two 4-Gy doses administered 2 weeks apart) *vs* the previously reported *D*BNCT protocol (8 Gy, in two 4-Gy doses administered 6 weeks apart) (Monti Hughes *et al*, 2011) and the fact that mucositis was only slight/moderate, we assessed the effect of a *single* application of BNCT at a dose of 8 Gy. Based on radiotoxicity in field-cancerized tissue, the feasibility of a second application would be considered. *S*BPA-BNCT and *S*[(GB-10+ BPA)-BNCT] at 8 Gy caused severe mucositis (Grade 5) in 100% of the animals treated with the BNCT protocols, with loss of pouch tissue. This precluded a second irradiation. Resolution of mucositis and T50 and T80 values were evaluated in the remaining portion of the pouch, conceivably exposed to lower dose levels as suggested by computational modelling studies (Farías *et al*, 2011). Mucositis resolved by the 4th week after irradiation. Table 2 shows that T50 and T80 were not reached within the follow-up period for the *S*BPA-BNCT and *S*[(GB-10+ BPA)-BNCT] protocols. *S*BO induced virtually no inhibitory effect. Severe mucositis (Grade 5) was also observed in normal pouch tissue.

Although the therapeutic effect of *S*BNCT (8 Gy) in the remaining portion of the pouch was enhanced compared with *D*BNCT (8 Gy in two 4-Gy doses administered 2 weeks apart) and *D*BNCT (8 Gy in two 4-Gy doses administered 6 weeks apart) (Monti Hughes *et al*, 2011), toxicity was severe. A computational analysis (Farías *et al*, 2011) revealed that the remaining portion of the pouch had received approximately 6 Gy absorbed dose. Based on these results, we performed a pilot study to explore the effect of a *S*BPA-BNCT protocol, prescribing 6 Gy absorbed dose. The pilot study was performed with the protocol known to induce the most severe toxicity, that is, BPA-BNCT. All 4 animals suffered Grade 5 mucositis and underwent tissue loss. This precluded a second irradiation. Mucositis resolution and inhibitory effect (in terms of T50 and T80 values) were evaluated analysing the remaining portion of the pouch, conceivably exposed to lower dose levels (Farías *et al*, 2011). Mucositis resolved by the 3rd week after irradiation. Table 2 shows that T50 for the *S*BPA-BNCT group was reached in approximately twice the time corresponding to the control group. T80 was not reached within the follow-up period of 8 months.

As toxicity was still severe for the *S*BPA-BNCT 6 Gy protocol, we reduced the dose further, taking into account the mean dose received by the remaining portion of the pouch (5 Gy) according to the corresponding computational studies (Farías *et al*, 2011). A new set of irradiations involved, tentatively and depending on toxicity, a *double* application of BNCT, at a total dose of 10 Gy in two 5-Gy doses administered 4 weeks apart. For the *D*BPA-BNCT protocol, 67% of the animals suffered Grade 5 mucositis, whereas for *D*(GB-10+ BPA)-BNCT, only 33% exhibited Grade 5 mucositis. *D*(GB-10+ BPA)-BNCT was the BNCT protocol that induced less severe

Table 2 T50 and T80 (time at which 50% and 80% of the animals exhibited tumour development from field-cancerized tissue) for each of the groups as indicated

Experimental group	*Protocol*	*T50*	*T80*
Control group (cancerized, sham-irradiated)		4 weeks	13 weeks
Double application (8 Gy in two 4-Gy doses administered 6 weeks apart)	*D*BO	8 weeks	20 weeks
[Previous study	*D*BPA-BNCT	8 weeks	Not reached
(Monti Hughes *et al*, 2011)]	*D*(GB-10+ BPA)-BNCT	15 weeks	18 weeks
Double application (8 Gy in two 4-Gy doses administered 2 weeks apart)	*D*BO	11 weeks	29 weeks
[This study]	*D*BPA-BNCT	6 weeks	15 weeks
	D(GB-10+ BPA)-BNCT	6 weeks	Not reached
Single application (8 Gy)	*S*BO	8 weeks	9 weeks
[This study]	*S*BPA-BNCT[a]	Not reached	Not reached
	S(GB-10+ BPA)-BNCT[a]	Not reached	Not reached
Single application (6 Gy)	*S*BPA-BNCT[a]	9 weeks	Not reached
[This study]			
Double application	*D*BO	3 weeks	7 weeks
(10 Gy in two 5-Gy doses administered 4 weeks apart)	*D*BPA-BNCT[a]	10 weeks	13 weeks
[This study]	*D*(GB-10+ BPA)-BNCT[a]	Not reached	Not reached

[a]In the case of tissue loss due to severe radiotoxicity, the data correspond to the remaining portion of the pouch.

mucositis in field-cancerized tissue. Although both BNCT protocols induced cases of severe mucositis (Grade 5) with some cases of tissue loss, the remaining portion of the pouch was long enough to perform a second irradiation in all cases. Mucositis seen after the second irradiation was not exacerbated by the first application and resolved by 6–8 weeks after the first application. T50 showed an inhibitory effect for the *D*BPA-BNCT protocol *vs* the control group and the *D*BO group. Conversely, T80 for *D*BPA-BNCT was similar to the control group. The *D* (GB-10+ BPA)-BNCT group did not reach T50 or T80 within the follow-up period of 8 months. As shown in Figure 1, it is noteworthy that this protocol induced an inhibitory effect of 100% up to 2 months of follow-up (whereas *D*BO and *D*BPA-BNCT induced a 100% inhibition up to 0.5 months of follow-up). No statistically significant differences were found between the *D*BO group and control throughout follow-up. Although *D*BPA-BNCT exhibited an inhibitory trend *vs* control up to 2 months, this difference did not reach statistical significance. *D*(GB-10+ BPA)-BNCT exhibited a highly statistically significant inhibitory effect *vs* control at 1 month ($P = 0.0264$), 2 months ($P = 0.0018$), 4 months ($P = 0.0103$) and at 6 and 8 months ($P = 0.0035$). At 8-month follow-up, the inhibitory effect on tumour development *vs* control persisted at 63%. This *long-term* inhibitory effect was associated with only slight toxicity in 67% of the cases. The *D*BNCT protocols induced only G0-G2 mucositis in normal tissue.

Discussion

The present study reports the results of a series of radiobiological BNCT experiments aimed at optimizing BNCT in terms of inhibiting tumour development in field-cancerized tissue and minimizing mucositis in this dose-limiting tissue in an experimental model of precancer. The clinical relevance of inhibiting tumour development from field-cancerized tissue while minimizing mucositis lies in the fact that recurrences and/or second primary tumours are frequently the cause of therapeutic failure (Ge *et al*, 2010) and that oral mucositis is a dose-limiting effect in conventional radiotherapy for head and neck tumours (Sonis, 2009) and BNCT for brain tumours and head and neck tumours (Kankaanranta *et al*, 2011, 2012).

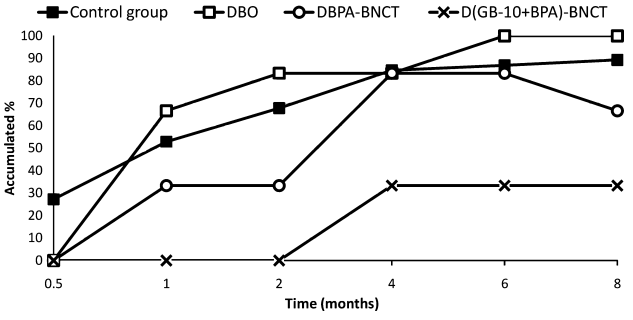

Figure 1 Accumulated percentage of animals that exhibited tumour development from field-cancerized tissue as a function of time post-T0 for the control group and each of the treatment groups corresponding to the *D*BNCT protocol at 10 Gy total dose, in two 5-Gy doses administered 4 weeks apart

Because targeting of all populations within a target tissue 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 (e.g. Ono *et al*, 1999; Trivillin *et al*, 2006; Heber *et al*, 2007). Within this context, we were particularly interested in exploring combined boron compound administration protocols herein. An additional asset of these protocols is that they employ the boron compounds BPA and GB-10, both approved for use in human subjects (Molinari *et al*, 2011). The fact that sodium mercaptoundecahydro-*closo*-dodecaborate (BSH) is being investigated clinically as a stand-alone boron agent for BNCT of brain tumours (e.g. Nakagawa *et al*, 2009) and in combination with BPA for recurrent head and neck malignancies (e.g. Kato *et al*, 2009) would make it a particularly interesting boron compound to explore in the future. A specific advantage of BNCT mediated by GB-10 is the fact that it induces remarkably mild mucositis in field-cancerized tissue at therapeutically useful doses (Trivillin *et al*, 2006). Particular attention should be paid to this aspect in future studies with BSH.

Our working hypothesis to test the *double* application of BNCT rather than to deliver the full dose with a *single* application was that dose fractionation would reduce BNCT toxicity in terms of mucositis in field-cancerized tissue (Molinari *et al*, 2011). Based on clinical trials for head and neck cancer (Kankaanranta *et al*, 2012) and our own experience, Grade 5 mucositis was considered severe toxicity in this model. Our findings showed that, in effect, the *double* application protocols caused less severe mucositis. It is known that tissues with a faster rate of basal cell proliferation are more liable to develop mucositis (Sonis *et al*, 2000). Thus, the reduction in DNA synthesis induced by BNCT previously described in hamster cheek pouch field-cancerized tissue (Heber *et al*, 2007) would make the tissue exposed to the second application of BNCT less or, at worst, equally liable to develop mucositis than if the total dose is delivered in a *single* application. These effects must be interpreted in the context of low and high LET radiation dose components of BNCT (Hopewell *et al*, 2011). Based on the known fact that mucositis is a multistage process initiated by mucosal injury and associated with an increased production of inflammatory cytokines which cause direct mucosal damage and initiate positive feedback loops (Mais, 2006), the interval between BNCT applications might conceivably allow the inflammatory process to partially subside before the second dose is delivered, precluding the exacerbation of mucositis. In terms of therapeutic efficacy, it is known that lengthening overall treatment time in conventional (low LET) radiotherapy reduces toxicity but also reduces tumour control probability (e.g. Dörr *et al*, 2005). However, in the case of BNCT in which the radiation dose is composed of a combination of high and low LET radiation components, a *double* application would allow for retargeting of cells that were refractory to the first

application (Molinari *et al*, 2011). Nevertheless, the present data showed that dose fractionation reduced therapeutic efficacy somewhat.

A pivotal aspect of *double* applications of BNCT is the time interval between applications. Our working hypothesis was that the shortest interval that did not result in severe mucositis would be the most therapeutically effective option because it would avoid repopulation as much as possible. However, the fact that in the case of the *double* application protocols with a 2-week interval, the beam-only protocol inhibited tumour development more than the BNCT protocols was unexpected and counterintuitive. The inflammatory process associated with moderate mucositis (G1-G3) in field-cancerized tissue induced by the BNCT protocols (*vs* G1 mucositis for the beam-only protocol) could favour tumour development. It is known that inflammation-induced tumour promotion can lead to the activation of premalignant lesions (Pérez *et al*, 2005; Lewis and Pollard, 2006; Grivennikov *et al*, 2010). Furthermore, chronic inflammation has been described as one of the hallmarks of cancer, acting on any stage of tumorigenesis (e.g. Multhoff and Radons, 2012). Within this context, mucositis would play a double role as an undesirable, dose-limiting side effect and as a tumorigenesis enhancer. Based on these findings, the working hypothesis that the best therapeutic advantage is always achieved by administering the most aggressive treatment (in terms of increasing irradiation dose or shortening the interval between double applications) that does not cause severe mucositis, should be revised for oral cancer.

The best therapeutic effect was afforded by a *double* application of BNCT mediated by GB-10+ BPA at a total dose of 10 Gy to field-cancerized tissue, in two 5-Gy doses administered 4 weeks apart. Inhibition of tumour development *vs* control was 100% up to 2 months post-treatment and persisted at 63% 8 months post-treatment. Mucositis was slight in the dose-limiting field-cancerized tissue in 67% of cases and was also slight in all cases of normal tissue. The data reported herein show that issues such as dose levels and dose fractionation, interval between applications and choice of boron compounds are pivotal to therapeutic advantage and must be tailored for a particular pathology and anatomic site. The present study determined treatment conditions that would contribute to optimize BNCT for precancer.

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Author contributions

AMH, VAT and AES designed the study. AMH was responsible for the experimental work and performed animal follow-up. ECCP, ST, and PC performed the irradiations and dosimetric calculations. ROF and SJG performed the computational modeling studies. MEI and RFA performed histopathological analyses. MM and DWN contributed to the dosimetric characterization of the RA-3 irradiation facility. AMH, ECCP, ST, MAG, EMH, AJM and VAT collaborated with different stages of the experimental work. AMH and AES drafted the manuscript. All authors participated in the discussion of the data and read and approved the final manuscript.

Conflict of interest

All authors declare no conflicts of interest.

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