Boron Nanoparticles Inhibit Tumour Growth by Boron Neutron Capture Therapy in the Murine B16-OVA Model

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Abstract. *Background: Boron neutron capture therapy usually relies on soluble, rather than particulate, boron compounds. This study evaluated the use of a novel boron nanoparticle for boron neutron capture therapy. Materials and Methods: Two hundred and fifty thousand B16-OVA tumour cells, pre-incubated with boron nanoparticles for 12 hours, were injected subcutaneously into C57BL/6J mice. The tumour sites were exposed to different doses of neutron radiation one, four, or eight days after tumour cell inoculation. Results: When the tumour site was irradiated with thermal neutrons one day after injection, tumour growth was delayed and the treated mice survived longer than untreated controls (median survival time 20 days (N=8) compared with 10 days (N=7) for untreated mice). Conclusion: Boron nanoparticles significantly delay the growth of an aggressive B16-OVA tumour in vivo by boron neutron capture therapy.*

Neutron capture therapy was first suggested in 1936 by Locher (1), and the principle has since been regarded as an appealing, but technically challenging approach to the treatment of cancer.

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Boron neutron capture therapy of cancer relies on the administration of boron compounds, based on the stable and innocuous boron-10 isotope, combined with neutron radiation. The neutron component of this therapy has little effect on normal or tumour tissue because thermal neutrons (<0.4 eV) have insufficient energy to damage tissue. However, when the boron-10 isotope is exposed to neutrons, the boron-10 nucleus captures a neutron and promptly emits an alpha particle while the resulting lithium-7 nucleus recoils. Both the recoil lithium ion and the alpha particle are classified as high linear energy transfer radiation, and are highly destructive to cells within a distance of approximately 12 mm.

The goal of any boron neutron capture therapy is to achieve a high concentration of boron-10 in the tumour while avoiding accumulation of boron-10 in the normal tissues. However, obtaining sufficient amounts of boron in the tumour tissue by systemic administration of soluble boron compounds has proved difficult (2). As an alternative to soluble boron, the possibility that boron nanoparticles might hold promise as future therapeutic agents was investigated.

Materials and Methods

Mice. Female C57BL/6J BomTac mice, 8-12 weeks old (Taconic M&B, Ry, Denmark), were housed under standard conditions at the animal facility, the Faculty of Health Sciences, University of Aarhus and later at the animal facility, NRG, Petten, Holland.

The Danish National Ethics Commitee and the local ethics commitee at NRG approved all the experiments.

Cell cultures. The B16-OVA cell line (3), derived from the aggressive B16 melanoma cell line, was cultured in Ultra-Culture (BioWhittaker, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml glutamine (Gibco, Life Technologies, Paisley, Scotland), and 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA) to inhibit cells that do not express the OVA construct. The tumour cells were maintained at 37˚C and 5% CO_2.

Colloidal boron nanoparticle preparation. Commercially available boron-10 enriched (>96 at. % , Eagle Picher Technologies, Quapaw, OK, USA) boron carbide was processed by ball milling in an argon atmosphere as described previously (4). The resulting boron carbide nanoparticles were successfully functionalised with lissamine and the TAT peptide sequence to promote cellular adhesion and uptake of the particles, as described previously (5). The concentration was adjusted to 10 mg/ml in water.

Subcutaneous tumour establishment. Where appropriate, colloidal boron nanoparticles were added to the cell culture medium at a final concentration of 40 μg/ml, and the cells were incubated for 12 hours. The tumour cells were then harvested using trypsin-ethylenediaminetetraacetic acid (Gibco), centrifuged, and washed once in RPMI1640 (Gibco). The cell concentration was adjusted to $5x10^6$ per ml in UltraCulture, and 50 μl ($2.5x10^5$ cells) was injected subcutaneously in the upper hind leg of the C57BL/6J mice anaesthetised with isoflurane (Abbott, North Chicago, IL, USA).

Experimental setup. The boron-containing nanoparticles were tested in four different experimental setups. In all the experiments, the tumours established by injection of $2.5x10^5$ B16-OVA cells alone were compared with with tumours established by injection of $2.5x10^5$ B16-OVA cells that had been incubated with boron nanoparticles for the final 12 hours of culture.

In the first experiment, the tumours were allowed to grow for one day after injection of the tumour cells. The tumours were then exposed to either 30 minutes or 15 minutes of thermal neutron irradiation, while leaving a control group of tumours untreated. In the second experiment, the tumours were allowed to grow for eight days before exposure to thermal neutron irradiation for either 30 minutes or 15 minutes, while leaving a third group of tumours untreated. The third experiment was a repetition of the first experiment, but restricted to either 30 minutes of irradiation or no irradiation. In the fourth experiment, the tumours were allowed to grow for four days, and were then either irradiated for 30 minutes or left untreated.

Irradiation of mice. Immediately before irradiation, the mice were sedated by intraperitoneal injection of meditomedin at a dose of 2 mg/kg. Ten minutes later, the mice were fixed in a specially designed "mouse rack" made from a 2.54 cm thick lithium-6-enriched (89%) plate to shield the mouse from whole-body irradiation. Two conically shaped holes with an inner diameter of 2.0 cm had been drilled in the shielding material to expose only the tumour-bearing hind legs of the mice. The mice were thus exposed to thermal neutron irradiation for either 15 minutes or 30 minutes at a reactor output of 30 kW using the Low flux reactor, Nuclear Research and Consultancy Group (NRG), Petten, Holland. Non-irradiated control mice were sedated and placed in the mouse rack for either 15 or 30 minutes, but the rack was not exposed to radiation.

Tumour measurements. The tumours were measured on a daily basis using digital callipers. The tumour volumes were calculated as V=0.52 x length x width x width. In all the experiments, the mice were sacrificed when any tumour dimension exceeded 1 cm, or when visibly discomforted by the tumour, regardless of size. Accordingly, we evaluated the outcome of the four experiments as a "survival study".

Inductively coupled plasma atomic emission spectroscopy (ICP-AES). To assess the amount of boron present in the treated tumours, representative tumours from each experiment were excised and analysed. The tumours were extirpated immediately before the mice would otherwise have been irradiated. Control tumours (*i.e.*, tumours without boron particles) were also included from two mice in each experiment. Finally, in each experiment, the boron content of a sample representing the injected mixture of tumour cells and boron particles was measured. The samples were digested in 1.5 ml digestion mix (897 ml/l HNO_3 65% ; 61 ml/l $HClO_4$ 70% ; 42 ml/l HF 40%) and a recovery standard (0.5 ml 40 ppm cobalt solution) was added. For inductively coupled plasma-atomic emission spectroscopy, the 249.773 nm emission line was chosen to measure boron. For a 4 ml sample, the detection limit (defined as the mean value of the background plus three standard deviations) was between 0.001 and 0.15 ppm. This allowed detection of between 0.04 and 0.6 ppm of boron in the tissue sample weighing 1 g. All measurements were made in triplicate with a coefficient of variation of less than 2% . Internal standard samples were measured with prompt gamma ray spectroscopy.

Neutron fluence rate measurements. To verify that a uniform dose of thermal neutrons was delivered to the tumour area, delayed gamma-ray neutron activation analysis of implanted gold wire was recorded. Gold wires with a diameter of 0.25 mm and an approximate length of 10 mm, were inserted into the tumours of two mice that had been killed by cervical dislocation immediately before implantation of the wire. The mice were placed one above the other in the same rack used for irradiation of the mice in the actual experiments. The mice were then irradiated at 30 kW for a total of 15,000 seconds. After irradiation, the gold wires were removed, cleaned, and counted by gamma-ray spectroscopy.

Statistics. STATA 9.1 (Stata Corp, College Station, TX, USA) was used for Kaplan-Meier survival analysis, and a log-rank test for equality of survivor functions was applied.

Results

Tumour growth measurements. Figure 1 illustrates the growth of individual tumours after exposure to thermal neutron irradiation one day after injection of tumour cells (*i.e.*, pooled data from experiment 1 and experiment 3).

If exposed to 30 minutes of thermal neutron irradiation, tumour growth was visibly delayed when the tumour cells had been incubated with boron-containing nanoparticles, compared with tumours that were exposed to the same radiation but did not contain boron (Figure 1a). A similar effect of the nanoparticles was noted when the radiation dose was reduced to 15 minutes (Figure 1b), but the number of mice in this experiment was insufficient for conclusive

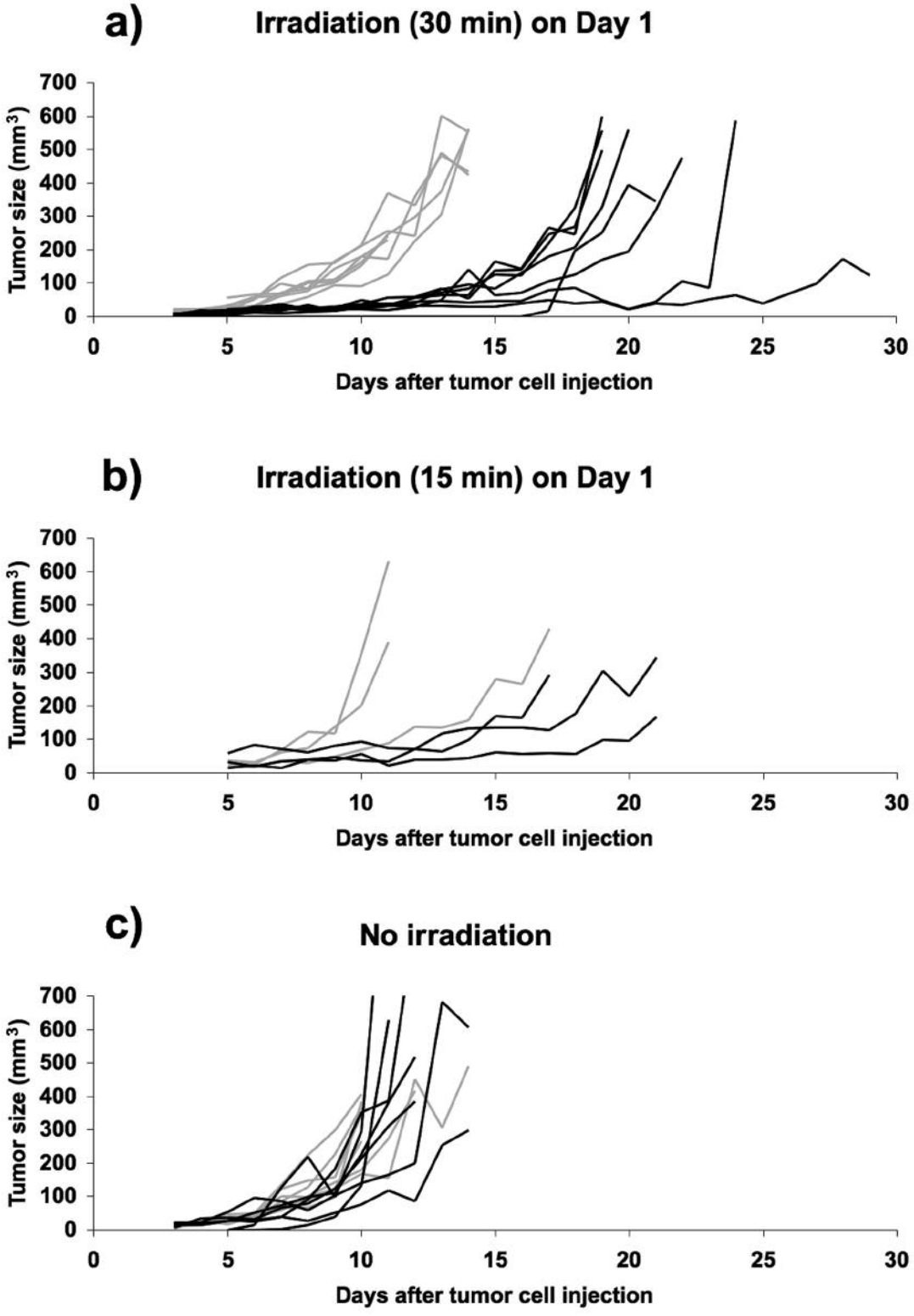

Figure 1. *Tumour growth after nanoparticle-mediated boron neutron capture therapy. Solid lines: Boron-containing tumours. Grey lines: Tumours without boron nanoparticles. a) Tumours irradiated for 30 minutes, 1 day after injection of tumour cells. b) Tumours irradiated for 15 minutes, 1 day after injection of tumour cells. c) Tumours not irradiated.*

statements. The boron nanoparticles had no effect if the tumours were not also irradiated by thermal neutrons (Figure 1c). Likewise, no effect of thermal neutron radiation was found in the absence of boron nanoparticles, regardless of whether the tumours were exposed to thermal neutron irradiation for 30 minutes or 15 minutes (data not shown).

When radiation was applied four or eight days after tumour-cell injection (experiment 2 and experiment 4, results not shown), no effect on tumour growth was seen regardless of the boron content of the tumours.

Mouse survival data. Figure 2a illustrates the effect of boron particles and 30 minutes of thermal neutron irradiation one day after the injection of tumour cells. The untreated mice (no boron, no irradiation) had a median survival-time of 10 days (n=7) after injection of the tumour cells. Boron nanoparticles in the tumours did not significantly (p=0.13) increase the median survival-time in the absence of thermal neutron irradiation (median survival-time 12 days, n=7). However, irradiation with thermal neutrons for 30 minutes in the absence of boron nanoparticles resulted in a marginally significant (p=0.018) increase in the median survival-time (median survival-time 14 days, n=7). The mice treated with a combination of boron nanoparticles and irradiation for 30 minutes had a highly significant increase in the median survival-time compared with both the untreated controls (p<0.00005) and the mice treated with radiation only (p=0.0002). In the group of mice receiving both boron and 30 minutes of radiation, the median survival time was 20 days (n=8).

Figure 2b illustrates the result of irradiating tumours for 15 minutes one day after the injection of tumour cells. Again, the combination of boron nanoparticles in the tumour and thermal neutron irradiation prolonged the median survival-time compared with no treatment, with boron alone and with radiation alone. However, the number of mice (n=3) was too low for meaningful statistical analysis.

When the tumours were irradiated four or eight days after injection of the tumour cells, boron particles had no statistically significant effect on the median survival time (data not shown).

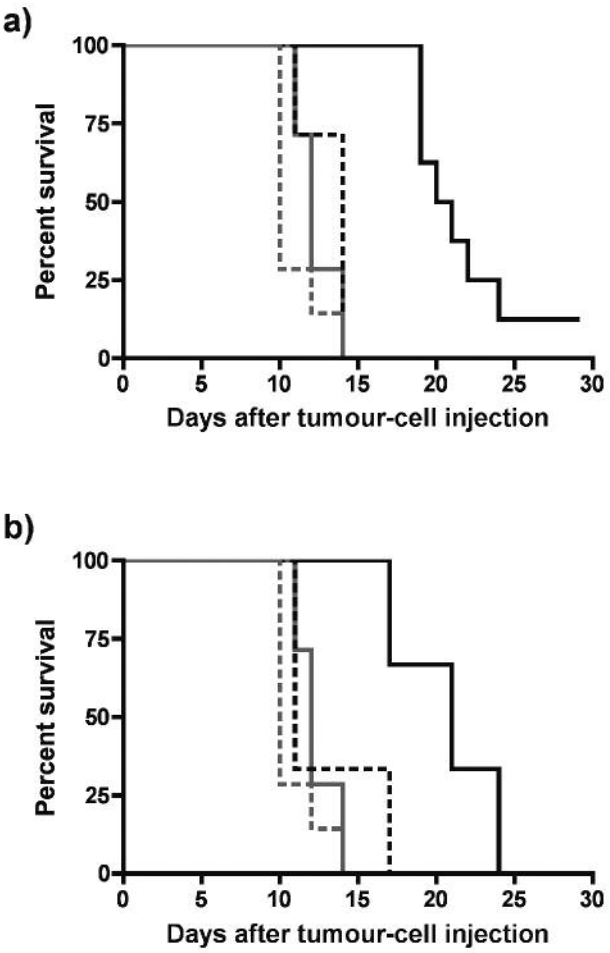

Figure 2. *a) Survival of mice irradiated 1 day after injection of tumour cells. Solid black line: mice with boron-containing tumours, irradiated for 30 minutes. Dotted black line: mice with no boron in tumours, irradiated for 30 minutes. Solid grey line: mice with boron-containing tumours, without irradiation. Dotted grey line: mice with no boron in tumours, without irradiation. b) Survival of mice irradiated 1 day after injection of tumour cells. Solid black line: mice with boron-containing tumours, irradiated for 15 minutes. Dotted black line: mice with no boron in tumours, irradiated for 15 minutes. Solid grey line: mice with boron-containing tumours, without irradiation. Dotted grey line: mice with no boron in tumours, without irradiation.*

Measurement of thermal neutron fluence rate. The thermal fluence rate (corrected for the contribution of epithermal neutrons and self-shielding) was 3.64×10^{12} m^{-2} s^{-2} for the first gold wire and 3.30×10^{12} m^{-2} s^{-2} for the second. The total uncertainty in the adjusted thermal fluence rate was estimated to 4% and included the uncertainty arising from the adjustment procedure.

Boron content of tumours. The boron content of all the samples, determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES), are presented in Table I. The measurements indicated that the boron remained largely associated with the tumour. Typically, 75% to 100% of the injected amount of boron remained in the tumour, even when the tumour had grown for eight days. The average boron content of the negative control-tumours was 0.1 μg (SD=0.21, N=8), reflecting the background signal in the ICP measurements.

Discussion

The combination of boron nanoparticles and exposure to thermal neutron radiation significantly delayed tumour growth (see Figure 1) and significantly prolonged mouse

Table I. *Measurement of boron content in tumours.*

	Tumour age (days)	Number of tumours analysed	Tumour weight (mg)		Boron content of tumour (μg)		Boron content of tumour (of injected amount)		Boron concentration (PPM)		Injected amount of boron (μg)
			Average	Standard deviation	Average	Standard deviation	Average	Standard deviation	Average	Standard deviation	
Experiment 1	1	5	**9.0**	4.3	**18.0**	4.7	**87**%	23%	**2426**	1190	**20.7**
Experiment 3	1	4	**7.0**	3.3	**9.3**	0.5	**104**%	6%	**1163**	709	**8.9**
Experiment 4	4	4	**12.5**	4.5	**5.3**	0.2	**88**%	4%	**407**	174	**6.0**
Experiment 2	8	5	**39.9**	19.3	**11.9**	1.9	**77**%	9%	**335**	102	**15.0**

survival (see Figure 2), whereas boron nanoparticles or radiation alone had little effect. These results were obtained in two independent experiments. As such, boron nanoparticles can, under certain conditions, affect the growth of a solid tumour by neutron capture.

However, tumour growth was only affected when the boron-containing tumours were irradiated the day after tumour-cell injection. When irradiation was postponed to four days or eight days after tumour-cell injection, the treatment failed to delay tumour growth.

The injected boron particles essentially remained in the tumour (see Table I). However, the total amount of injected boron was higher in the first two experiments than in the last two (20.7 μg and 15.0 μg *versus* 8.9 μg and 6.0 μg, respectively). This fact may reflect a batch-to-batch variation in the boron nanoparticle preparation. Reducing the initial amount of boron in the injected mixture would be expected to lower the effect of thermal neutron irradiation accordingly. However, in both Experiment 1 (20.7 μg boron) and Experiment 3 (8.9 μg boron), similar therapeutic effects of boron combined with thermal neutron irradiation were observed. Evidently, 8.9 mg boron nanoparticles was sufficient to delay tumour growth under the given conditions.

Although the murine model used in this study is artificial, in the sense that the boron particles and the tumour cells were mixed before subcutaneous injection, the results encourage further study of boron nanoparticles as therapeutic agents. Specifically, boron nanoparticles could readily be modified with "homing molecules" that would permit intravenous injection of the particles and subsequent tumour-specific accumulation. Several strategies for tumour-specific targeting of particles and compounds have been proposed. For example, lectins expressed by tumour cells have successfully been used as targets for lectin-binding avidin conjugated with a fluorescent molecule (6). Other receptors, such as vascular endothelial growth factor receptor 2 (VEGFR-2) expressed by growing endothelial cells during angiogenesis, have been used as targets of toxic fusion proteins (7). Also, folate-coupled nanoparticles have been presented as possible agents for imaging of folate-receptor positive tumours (8) and for intracellular delivery of DNA-particles (9).

A radically different approach would be to culture tumour-specific T cells *in vitro*, load the T cells with boron nanoparticles (10), and inject the T cells intravenously. By "concealing" the boron particles inside living, tumour-homing T cells, the particles might be less prone to entrapment and clearance by the reticuloendothelial system, as might otherwise be the fate of injected particles. We have previously demonstrated that OVA-specific $CD8^+$ T cells, cultured *in vitro* and injected into tumour-bearing mice, specifically accumulate in B16-OVA (expressing the OVA antigen) (11, 12). Tumour-specific T cells loaded with boron nanoparticles could therefore also be expected to infiltrate tumour tissue as part of the natural T-cell activity. Having reached the tumour, the T cell would be likely to die (certainly after exposure to thermal neutron radiation), thereby releasing the boron particle into the tumour lesion. Indeed, we are currently investigating the ability of T cells to function as transporters of nanoparticles to tumours.

In conclusion, boron nanoparticles can affect the growth of an aggressive B16-OVA melanoma *in vivo* by boron neutron capture therapy. The results of this initial study are sufficiently encouraging to continue research on the use of boron nanoparticles as a possible therapeutic agent in neutron capture therapy.

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