Therapeutic Efficacy for Hepatocellular Carcinoma by Boric Acid-mediated Boron Neutron Capture Therapy in a Rat Model

SY-YU LIN[1], CHEN-JOU LIN[2], JIUNN-WANG LIAO[3], JINN-JER PEIR[4], WEI-LIN CHEN[1], CHIN-WEN CHI[5,6], YUNG-CHANG LIN[7], YU-MING LIU[8] and FONG-IN CHOU[1,4]

[1]*Institute of Nuclear Engineering and Science and* [4]*Nuclear Science and Technology Development Center, National Tsing Hua University, Hsinchu, Taiwan, R.O.C.;*
[2]*Veterinary Medical Teaching Hospital,* [7]*Department of Veterinary Medicine and*
[3]*Graduate Institute of Veterinary Pathobiology, National Chung Hsing University, Taichung, Taiwan, R.O.C.;*
[5]*Department of Medical Research and Education, and* [8]*Cancer Center, Taipei Veterans General Hospital, Taipei, Taiwan, R.O.C.;*
[6]*Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan, R.O.C.*

Abstract. *Background: Hepatocellular carcinoma (HCC) is a common malignant tumor with poor prognosis. Boron neutron capture therapy (BNCT) may provide an alternative therapy for HCC. This study investigated the therapeutic efficacy of boric acid (BA)-mediated BNCT for HCC in a rat model. Materials and Methods: The pharmacokinetic and biodistribution of BA in N1S1 tumor-bearing rats were analyzed. Rats were injected with 25 mg B/kg body weight via tail veins before neutron irradiation at the Tsing Hua Open-pool Reactor, and the efficacy of BNCT was evaluated from the tumor size, tumor blood flow, and biochemical analyses. Results: HCC-bearing rats administered BNCT showed reductions in tumor size on ultrasound imaging, as well as an obvious reduction in the distribution of tumor blood flow. The lesion located in livers had disappeared on the 80th day after BNCT; a recovery of values to normal levels was also recorded. Conclusion: BA-mediated BNCT is a promising alternative for liver cancer therapy since the present study demonstrated the feasibility of curing a liver tumor and restoring liver function in rats. Efforts are underway to investigate the histopathological features and the detailed mechanisms of BA-mediated BNCT.*

Hepatocellular carcinoma (HCC) is one of the most common types of cancer in Southeast Asia, Africa, and Southern Europe (1, 2). It is also the second most common cancer in males and females in Taiwan (3). Surgery is the best treatment for patients with a focal liver tumor. However, HCC is usually diagnosed late in the course of the disease, and at the time of diagnosis, more than 80% of patients have vascular invasion and multifocal tumors (4, 5). The multi-port irradiation that is usually utilized in HCC treatment delivers a radiation dose that exceeds that which can be tolerated by normal liver, and may cause fatal liver failure (6, 7). The most important issues in this field are the improvement of therapeutic efficacy and reduction of complications. An effective treatment method should be non-invasive and have few side-effects; it should also have minimal effects on normal liver tissue and allow for the rapid recovery of patients. Boron neutron capture therapy (BNCT) may be effective in reaching these goals. It involves treating tumors with high linear-energy-transfer (LET) alpha and ^{7}Li particles, resulting in significant damage to tumor cells from the nuclear reaction of $^{10}B(n, \alpha)^{7}Li$ (8). High LET means that irradiation has a high energy density, which breaks the double strands of DNA, and the use of short-range radiation ensures that adjacent normal tissues are spared from radiation-induced damage. However, BNCT does not satisfy the aforesaid requirements because boron drugs for use in the treatment of HCC are lacking. Certain criteria need to be considered for the BNCT treatment of HCC, including the low-toxicity of the boron drug, its high retention in the HCC tumor and its vessels but low retention in the liver and adjacent normal tissues, and a sufficient thermal neutron fluency. Consequently, searching for an appropriate boron drug is the main aim in research on BNCT treatment for HCC at the Tsing Hua Open-pool Reactor, which can provide sufficient thermal neutron fluency (9).

Correspondence to: Professor Fong-In Chou, Ph.D., Nuclear Science and Technology Development Center, National Tsing Hua University, No.101, Section 2, Kuang-Fu Road, Hsinchu, Taiwan, 30013, R.O.C. Tel: +886 35742884, Fax: +886 35725974, e-mail: fichou@mx.nthu.edu.tw

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Borocaptate sodium (BSH) and boronophenylalanine (BPA) are the two boron drugs that are currently used for clinical BNCT (9, 10). BSH is a water-soluble boron drug that has been used in clinical trials of BNCT for patients with glioma. However, the disadvantage of BSH-mediated BNCT for treating HCC is low tumor to liver ratio of boron concentration (11). The disadvantage of BPA-mediated BNCT in treating HCC is that adjacent tissue accumulates five to ten times more boron than the tumor. According to animal studies in which BPA was used as the boron drug, the pancreas, which is adjacent to the liver, accumulates a higher concentration of boron than does the liver tumor (12, 13). Therefore, under neutron irradiation, when the liver tumor acquires a dose sufficient for BNCT treatment, the pancreas is severely damaged. Consequently, when BPA was used as the boron drug in a clinical trial of BNCT for the treatment of a liver tumor, the patient's liver had to be removed by surgery following drug injection to enable *ex vivo* neutron irradiation of the liver tumor. The liver was then autotransplanted back to the patient following neutron irradiation. This novel liver tumor therapy resulted in tumor complete response (14).

However, such *ex vivo* neutron irradiation has certain limitations and risks. For example, its success depends on the technique used by the surgeon to remove the organ, the physical condition of the patient, and the efficacy of sterilization throughout the surgery. *In vivo* BNCT for liver tumor, which does not require for removal of the liver from the body, may be preferable. The first attempt to use BNCT *in vivo* to treat HCC has been made. Multiple tumors in the right liver lobe were treated with BNCT, which involved the intra-arterial administration of BPA and BSH through a catheter located in the right hepatic artery, followed by the injection of a mixed BSH/lipiodol emulsion. Tumors in the left lobe were treated with hepatic arterial chemoembolization. This method has significantly lower risk than that of *ex vivo* BNCT treatment for HCC. However, BNCT-treated tumors tend to recur 3.5 months after BNCT and the patient died from liver dysfunction 10 months after BNCT (15). Hence, new boron drugs for BNCT for the treatment of HCC need be developed.

Boric acid (BA; H_3BO_3) is a small, uncharged molecule, with a molecular weight of 61.83 g. In living organisms, BA is rapidly and passively transported and diffuses throughout the body into cells along with the body fluids (16, 17). Since BA does not accumulate in the soft tissues, it is commonly used as an internal reference in BNCT studies to assess the distribution of boron drugs, or as an internal standard for determining boron concentrations (18, 19). With respect to its use in BNCT, radiation-induced complications of organs adjacent to tumor regions must be evaluated.

In our investigation, BA was used as the boron-containing compound to evaluate the biological effects of radiation on normal liver tissue and adjacent organs in a hepatoma-bearing rat model under BNCT (20). This research unexpectedly demonstrated that when an aqueous solution of ^{10}B-BA is used as the only boron drug administered to hepatoma-bearing rats, no clear damage to the normal liver is observed after BNCT and the liver tumor is eliminated.

Materials and Methods

Preparation of BA solution. ^{10}B-Enriched BA (99% ^{10}B), purchased from Aldrich Inc. (Darmstadt, Germany), was used as a boron drug in BNCT for treating liver cancer. BA used in the experiment comprises 99% of ^{10}B, and 1% of ^{11}B. The BA solution was prepared by adding an adequate amount of BA powder to a normal saline solution to yield the required ^{10}B concentration. The pH of the BA solution was adjusted to 7.2-7.3; the solution was then sterilized using a 0.22 μm filter and stored as a stock solution (6000 μg/ml) at 4˚C until use.

Measurement of boron concentration by inductively coupled plasma atomic emission spectrometer (ICP-AES). Atomic emission measurements require that the sample is dissolved before analysis. For microwave digestion, the samples were initially placed in a Teflon high-pressure digestion vessel, and then 3 ml of concentrated nitric acid (14 N, 65%) and 0.5 ml of hydrogen peroxide (30-35%) were added. The vessel was sealed before being placed into a microwave digestion system (MLS 1200 Milestone, Italy) for decomposition. The decomposition proceeded in two stages; the first stage of digestion took 3 min with the power set at 300 W, and the second stage of digestion took 2 min with the power set at 600 W. Finally, the sample was allowed to cool and depressurize for 20 min. The sample subsequently became a clear solution upon complete digestion. After the sample was diluted with deionized water, an ICP-AES (OPTIMA 2000 DV; PerkinElmer Instruments, Norwalk, CT, USA) was used to measure the boron concentration therein. For the analysis, the temperature of the argon plasma was set at 6000-7000 K; the analytical wavelength was set at 249.773 nm, and the rate of liquid uptake was set at approximately 2 ml/min; the operating voltage after the formation of plasma was set at 40 V (21).

Establishment of liver tumor-bearing animal model. Tumor cell culture: The N1S1 rat hepatoma cell line (CRl-1604; American Type Culture Collection, Rockville, MD, USA) was used in the experiments. The N1S1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY, USA) that was supplemented with L-glutamine (2 mM) and a mixture of penicillin (100 IU/ml) and streptomycin (100 μg/ml) antibiotics. The pH of the medium was adjusted to 7.2. Subsequently, heat-inactivated fetal bovine serum (10% v/v) was added to the medium, which was then filtered through a 0.2 μm membrane. The medium was stored at 4˚C before use. N1S1 cells were maintained in suspension in culture flasks at 37˚C in a humidified atmosphere that contained 5% CO_2 until they were required for inoculation (22).

Implantation of tumor cells into the livers of Sprague-Dawley (SD) rats: Approval was obtained from the Institutional Animal Care and Use Committee of National Tsing Hua University, Taiwan (Approval No 09945). Male SD rats weighed 200-250 g were obtained from the BioLASCO Taiwan Co., Ltd (Taipei, Taiwan) and used for the liver tumor-bearing animal model in the experiments. The SD rats were bred in an animal room at 22±3˚C and a relative humidity of 40%-70% with a 12-hour light-dark cycle. Animal feed

and water were unlimited, and feeding patterns were monitored. For the purpose of tumor implantation, before anesthesia, the SD rats were subcutaneously injected with 0.1 mg/kg bw of Atropine to inhibit secretion of the respiratory tract and the salivary gland, and prevent bradycardia by overactivity of the vagus nerve. Ten to 15 minutes after atropine had been administered to the rats, the rats were intraperitoneally injected with 20-40 mg/kg bw of Zoletil 50 and 10 mg/kg bw of xylazine to sedate them. After the anesthesia, subxiphoid incision was performed to allow tumor cell implantation. N1S1 cells (6×10^6 cell/0.1 ml suspension) were directly and slowly injected into the hepatic lobe of each rat (22, 23). Following the injection, a cotton swab was used to compress the puncture site for at least one minute to prevent infusion leakage and bleeding. Finally, the abdomen of the rats was closed layer by layer. The rats were then released back into the cage, and their activities and survival were continuously monitored. After injection of the tumor cells, an ultrasound scan was conducted to monitor the growth and size of the tumor and the distribution of blood vessels around and in it (24, 25).

Pharmacokinetic analysis of BA in liver tumor-bearing SD rats. BA was administered to the rats *via* tail vein. The dose was 25 mg ^{10}B/kg bw. Blood samples were drawn from the animals before and after administration to measure the concentration of boron in the blood in order to determine its pharmacokinetics in the rats. The blood sample was stored in a heparin tube and uniformly mixed with an equal amount of 2.5% Triton X-100 before being diluted by adding 1% nitric acid, which allowed the boron concentrations of the samples to be analyzed using ICP-AES (21). The relationship between the mean boron concentration and time is presented as a semi-log plot using a two-compartment model (26) (Figure 1).

Analysis of biodistribution of BA in tumor-bearing SD rats. Boric acid (25 mg ^{10}B/kg bw) was administered to the liver tumor-bearing rats *via* tail vein. The rats were sacrificed at 30 min, 1 h, 2 h, and 4 h after the administration of the drug; the average diameter of the tumors was 1-1.5 cm. Central and peripheral parts of the tumor, normal liver tissue from the tumor-bearing lobe, normal liver tissue from the normal lobe, lung, kidney, heart, intestine, pancreas, spleen, stomach, testis, and muscle tissue were collected, and then digested in a microwave digestion system for subsequent analysis of the boron concentrations in the tissues using ICP-AES. The samples were weighed before digestion. The samples underwent microwave digestion as described above. The resulting solutions were confirmed to be completely dissolved after they had become clear, and the solutions obtained were diluted by the addition of deionized water.

BNCT treatment. Rats that had been implanted with the N1S1 tumor cells were subjected to BNCT treatment on the 17th day after the implantation. BNCT was carried out at the Tsing Hua Open-pool Reactor at the National Tsing Hua University, Taiwan. The beam aperture for BNCT had a diameter of 14 cm. During the BNCT treatment, the reactor power was 1.2 MW, and the thermal, epithermal and fast neutron fluxes were 1.34×10^8, 1.07×10^9, and 7.66×10^7 neutrons cm^{-2} s^{-1} respectively, at the beam aperture (27). SD rats were randomly divided into three groups: a group of seven normal, untreated rats; a tumor comparison group of seven rats that had received the implanted tumor cells but no BNCT; and a BNCT treatment group of three subgroups, each of which comprised seven rats. The Monte Carlo N-Particle Transport Code (MCNP) was utilized to calculate the physical doses to be used to treat the tumors. The tumors in the G1 subgroup received a dose of 11.18 Gy; those in the G2 subgroup received a dose of 7.97 Gy; and those in the G3 subgroup received a dose of 5.61 Gy. Before receiving neutron irradiation, each of the liver tumor-bearing rats was injected with 25 mg B/kg of BA *via* tail vein. At 15 min before irradiation, the rats were injected with xylazine and Zoletil for anesthesia. Thirty minutes following the injection, two of the rats were placed behind the collimator, with their liver tumors aligned with the aperture of the collimator; they then received irradiation for different periods such that they received different doses of radiation.

Evaluation of therapeutic efficacy. The efficacy of BNCT was evaluated from the size of the tumor, blood flow of the tumor, and biochemical analysis. The follow-up interval was one day before BNCT to 80 days after BNCT.

Ultrasound examinations: All abdominal ultrasonography examinations were performed by one of the authors (CJ Lin) using the same ultrasound machine (Xario ultrasound unit; Toshiba, Tokyo, Japan), and multifrequency linear (PLT-1204BT, 12 MHz) probe. Well-defined, round or oval, hyperechogenic nodular lesions within the liver were identified in the liver tumor-bearing rats. The tumor lesions were visible in both longitudinal and transverse scans, being more easily imaged in transverse scans. The image was captured on the day before BNCT treatment (or on the 17th day following injection of the tumor cells) and on days 4, 8, 11, 18, 25, 30, 60 and 80 following BNCT treatment. The initial size of the tumor was determined from the maximum transverse (A) and sagittal dimensions (B) of the lesion. The proportional reduction of tumor size was calculated using the following equation:

$$x = \frac{(A \times B) - (A' \times B')}{(A \times B)} \times 100\%$$

where A and B represent the major axes of a sagittal section and a transverse section, respectively from a scan that was performed on the day before BNCT treatment, and *A'* and *B'* represent the major axes of a sagittal section and a transverse section at specific time intervals following the BNCT treatment. The initial size of a tumor on the day before BNCT treatment was x=0; a negative value of x indicates an increase in the tumor size, and a positive value indicates a decrease in the tumor size. A value of 1 indicates that the tumor has completely vanished and so the ultimate aim has been achieved (28). In the experiments, 3D Doppler imaging ultrasound scanner was used to detect the distribution of tumor blood flow in rats that had undergone BNCT (28).

Biochemical analysis: A biochemical analysis of serum was performed to evaluate liver and kidney functions. All blood was collected from the tail vein. The blood was centrifuged for 15 min at 936 ×*g* and plasma was collected to perform a biochemical analysis of the serum of the rat using a serum biochemical analyzer (Chiron Diagnostics Corporation, Oberlin, OH, USA). The parameters in the analysis included the liver function enzymes, glutamate oxaloacetate transaminase (GOT) and glutamate pyurvate transaminase (GPT) (29, 30), and blood urea nitrogen (BUN) for kidney function assessment.

Statistical analyses. Data are shown as means with standard deviations of grouped data (mean±SD). The data for the experimental groups were compared using Student's *t*-test, in which a *p*-value of less than 0.05 indicated a statistically significant difference.

Table I. *Biodistribution of boric acid in rats at various times following administration.*

Organ	Boron concentrations (μg/ml)			
	0.5 h (n=7)	1 h (n=5)	2 h (n=5)	4 h (n=3)
Tumor (center)	28.56±7.42	20.47±0.99	18.57±4.03	10.95±0.70
Tumor (peripheral)	31.91±5.28	21.03±2.46	19.15±3.83	10.25±1.22
Lobe with tumor from a normal liver	23.11±3.19	19.11±1.02	17.98±3.79	10.54±1.78
Normal lobe from a normal liver	21.24±4.07	18.82±0.68	17.53±4.51	10.58±1.19
Lung	22.78±2.61	18.50±1.95	17.54±3.35	11.60±3.08
Heart	22.83±0.62	17.78±0.45	18.75±2.95	11.08±1.29
Spleen	22.72±3.32	20.25±2.72	18.80±3.31	12.60±4.05
Pancreas	19.92±2.48	15.97±0.97	14.55±2.85	8.29±0.74
Kidneys	42.36±8.54***	35.32±9.61*	24.90±5.28*	16.81±1.00
Stomach	20.55±2.91	20.06±4.76	17.29±2.95	10.40±0.69
Intestine	23.88±3.41	18.90±2.71	17.35±2.80	9.94±0.41
Testes	15.48±3.41*	16.04±4.86	17.83±0.86	11.77±0.61
Muscle	22.76±3.44	20.76±3.96	16.81±4.42	10.26±0.37
Blood	25.75±3.24	22.11±2.13	18.46±2.35	11.38±1.12

Boric acid was administered to the rats *via* tail veins at 25 mg B/kg bw. Data are mean±SD. (*p<0.05, ***p<0.001 *vs.* other organs).

Results

Pharmacokinetics of BA in tumor-bearing SD rats. The acute toxicity of BA in SD rats administered by single-dose intravenous injection was investigated. This investigation was based on the Biological Evaluation of Medical Devices, Part 11: Tests for systemic toxicity, ISO 10993-11: 2006, with the slight modification that was performed by one of the authors (JW Liao) at the Graduate Institute of Veterinary Pathobiology, National Chung Hsing University, Taiwan. Experimental results on the acute single-dose toxicity of BA revealed no observable adverse effect of BA *via* the single intravenous route in rats at a concentration of 400 mg/kg (data not shown). In this investigation, 150 mg/kg bw of BA contained a boron dose of 25 mg/kg bw was used. A liver tumor-bearing SD rat was intravenously injected with BA (25 mg B/kg bw) *via* the tail, and blood samples were drawn to analyze the boron concentration. Figure 1 shows the boron concentration in the blood of the rats at different times after BA injection. The curve of the pharmacokinetics consists of two parts, out of which the first exhibits a sudden drop in concentration within 20 min of the injection, and the second shows a slowly declining concentration. BA was metabolized as indicated by a double exponential curve, and the two zones are associated with the fast distribution phase and the slow excretion phase. The initial blood boron concentration detected at 1 min after BA injection was 104.6±36.23 μg/ml, which rapidly fell to 28.92±3.73 μg/ml within 20 min, before steadily decreasing thereafter; it had dropped to 8.49±1.62 μg/ml by 360 min after the injection. Data are means±SD.

Biodistribution of BA in tumor-bearing SD rats. Table I presents the measured boron concentrations in the tumors and the soft tissue of the liver tumor-bearing SD rats 0.5, 1, 2, and 4 h following intravenous injection with BA (25 mg B/kg bw). Boron concentrations decreased gradually over the four hours of investigation; only in the testes, did the boron concentration increase up to 2 h, and decrease thereafter. At 0.5 h after the injection of BA, the ratio of boron concentration in the tumors to that in the normal liver, heart, lungs, pancreas, and stomach reached 1.4 to 1.5. The boron concentration in normal tissues other than the kidneys was less than that in the tumor tissue. The boron concentration in the liver tumors was higher than that in the normal liver. The boron concentration in the peripheral parts of the liver tumors slightly exceeded that in the central tumor tissues. The difference between the boron concentrations in the tumor and the normal liver tissue was negligible 4 h after the injection.

Changes in body weight of rats after BNCT. The BNCT treatment group was divided into three subgroups in which the tumors received different doses of radiation. The tumors in the G1 subgroup received a dose of 11.18 Gy; those in the G2 subgroup received a dose of 7.97 Gy, and those in the G3 subgroup received a dose of 5.61 Gy. The rats that underwent BNCT treatment exhibited clear weight loss 3-10 days after they were treated. The rats in the subgroups reached their maximum weight loss within different periods. The rats of G1 reached their lowest weight, 78% of their initial weight, eight days after irradiation; the rats of G2 reached their lowest weight, 89% of the initial weight, four days after irradiation, and the rats of G3 reached their lowest weight, 92% of the

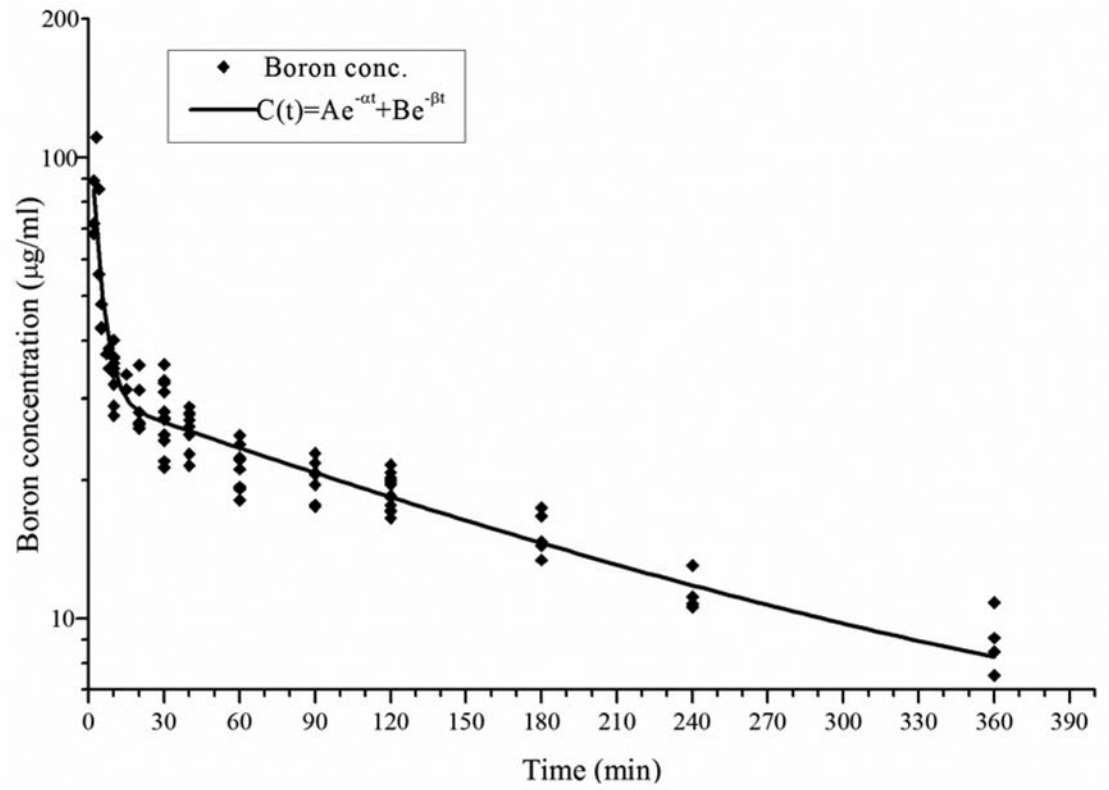

Figure 1. *Concentrations of boron in blood of rats at different time intervals after injection of boric acid (n=3-12). A: The concentration of the drug in the central chamber; B: The concentration of the drug in the tissues; α: The half-life of the drug distribution; β: The half-life of the drug excretion; C(t): Concentration of boron at time t.*

initial weight, three days after irradiation. The rats suffered slight diarrhea and loss of appetite, but those that received lower doses exhibited faster weight recovery. The BNCT-treated rats all exhibited gradual weight recovery after reaching their lowest weight. The body weight of the tumor-bearing control rats remained constant day 17 to day 35 after the implantation of tumor cells and began to decrease thereafter. Figure 2 shows the dose-related changes in the body weights of rats after three doses of BNCT; the results reveal that the weight loss after irradiation was a temporary side-effect of BNCT, and the weight was recovered thereafter.

Biochemical changes in serum of rats following BNCT. Changes in biochemical parameters GOT, GPT and BUN were observed in the normal group, the tumor comparison group, and the three BNCT-treated subgroups of tumor-bearing rats. Table II presents the changes in the GOT, GPT and BUN values of rats before the day of, and on days 11, 30, and 80 after, BNCT treatment.

Before the implantation of tumor cells, the serum biochemical parameters (GOT, GPT and BUN) values of the rats in all groups were within their normal ranges. Experimental results revealed that, on the day before BNCT, the GOT values in tumor-bearing rats clearly exceeded those in the rats of the normal group. However, 80 days after BNCT treatment, the GOT values decreased almost to the normal range in all of the BNCT-treated subgroups which received different doses of radiation, and increased continuously in the rats of the tumor comparison group. The GPT values also increased in rats of the tumor comparison group, but returned to normal levels in rats of all the BNCT subgroups. In the experiments, the kidney function of rats was assessed by monitoring the changes in the BUN values. The BNCT-treated groups did not exhibit any meaningful change in BUN during the 80 days following BNCT treatment, but the tumor comparison group exhibited a clear increase in BUN 47 days following the implantation of the tumor cells.

Therapeutic efficacy of BA-mediated BNCT. After a settling time of one week, the SD rats underwent laparatomy to implant the tumor cells in the liver. The rats exhibited slight weight loss following surgery, but their weights recovered steadily in one to two days. Seventeen days after tumor implantation, the size of the tumor was checked by ultrasound scanning; the following day, the rat underwent BNCT treatment in THOR.

Table II. *Effects of boron neutron capture therapy (BNCT) on hepatic and kidney function indices in rats.*

Time (day)	Normal group	Tumor comparison group	BNCT treatment groups		
			G1	G2	G3
GOT (AST, the normal range is 83-118 U/l)					
–1[#]	118±56	250±130	250±148	271±39	246±89
11	106±40	319±76	202±127	204±9	180±8
30	106±26	419±316	111±29	144±20	153±3
80	97±12	539±128	127±8	116±8	133±41
GPT (ALT, the normal range is 35-47 U/l)					
–1	37±6	54±8	54±7	56±9	50±10
11	43±1	60±9	49±13	45±15	53±4
30	49±12	66±13	50±8	52±9	53±7
80	45±1	67±14	51±9	54±7	51±12
BUN (the normal range is 11-17 mg/dl)					
–1	14±3	16±2	16±2	13±3	14±1
11	16±2	16±2	15±3	13±2	15±3
30	18±4	22±4	14±2	16±5	14±3
80	14±3	24±3	17±3	16±2	14±4

BNCT-treated group was divided into G1, G2, and G3 subgroups receiving physical doses of 11.18 Gy, 7.97 Gy, and 5.61 Gy, respectively. Each subgroup comprised of seven rats. GOT: Glutamate oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase, BUN: blood urea nitrogen. [#]–1: Day before BNCT treatment.

Reduction of tumor size following BNCT. The liver tumor-bearing rats were scanned with ultrasound to determine the reduction in tumor size. Table III presents the tumor-reduction ratio for rats between one day before and 80 days following BNCT treatment. By the 11th day following BNCT treatment, the tumors in the three subgroups had shrunk significantly from their sizes before treatment ($p<0.001$). On day 30 following BNCT treatment, the tumor reduction ratios had increased. Accordingly, the tumor sizes in the three subgroups were 23% or less of the original tumor sizes. In the G1 subgroup, the tumor was only 6% of its original size. The reduction in tumor size gradually slowed down after 30 to 60 days following BNCT treatment, and continuous monitoring of the tumors until 80 days following the BNCT treatment did not reveal any new growth or recurrence. In contrast, the tumor size in the tumor comparison group of the rats had increased by 1.34 times.

Changes in tumor blood flow in BNCT-treated rats. Colour Doppler imaging analysis was performed to monitor the variety of processes associated with vessel flow of tumor on the day before BNCT treatment, and on the 8th, 12th, and 18th days following BNCT treatment. Figure 3 displays the Doppler images of tumor blood flow in the tumor comparison group, and the BNCT-treated G1, G2, and G3 subgroups. It shows that the liver tumors had extensive vasculature, and that the tumor blood flow clearly changed in the rats that underwent BNCT. Following BNCT treatment, blood flow in the tumor and its surroundings gradually decreased until it was undetectable. This effect was especially significant on the 8th day following treatment, when the decrease in the blood flow was the most pronounced. The blood flow in the tumor continued to decrease thereafter but more slowly. However, the relationship between the decrease in the tumor blood flow and the BNCT dose was not significant, and the doses administered to the three subgroups were determined all to have caused irreparable damage to the blood vessels. Although the blood flow around the tumor was clearly reduced, but the blood flow in normal liver region seemed unaffected. In contrast, the Doppler images revealed that the blood flow in the tumor of the tumor comparison group gradually increased over time.

Morphology of tumor-bearing liver after BNCT. Figure 4 displays the gross morphology of the liver of tumor-bearing rats on the 80th day after BNCT treatment. Large tumor masses were observed in the left lobe of liver in the tumor comparison group, but no tumor mass was found on the surface of the liver in any of the BNCT-treated groups. The upper panel of Figure 4 displays photographs of three livers from the G1, G2, and G3 BNCT-treated subgroups, which received 11.18, 7.97, and 5.61 Gy of radiation doses, respectively. No lesions were visible on the liver surfaces in the rats of any of the BNCT-treated subgroups. The lower panel of Figure 4 displays three photographs of livers from the tumor comparison group that were obtained at the same time. A tumor lump larger than 1.5 cm was observed on the left liver lobe of rat in the tumor comparison group. However, on day 80 after BNCT treatment, no difference was observed in the liver normal tissue of the rats in the BNCT-treated subgroups, and the tumor locations were no longer visible to the naked eye on the surface of the livers (although they remained visible under ultrasound scanning as an apparent hyperechoic spot in the liver).

Discussion

In this investigation, an aqueous solution of ^{10}B-BA was used as the boron drug for BNCT. Liver tumors were selectively destroyed by BNCT treatment in N1S1 liver tumor-bearing rats. Conventionally, successful BNCT requires a boron concentration in the tumor that is three times that in normal tissue, such that BNCT treatment can effectively kill the tumor cells while keeping damage to

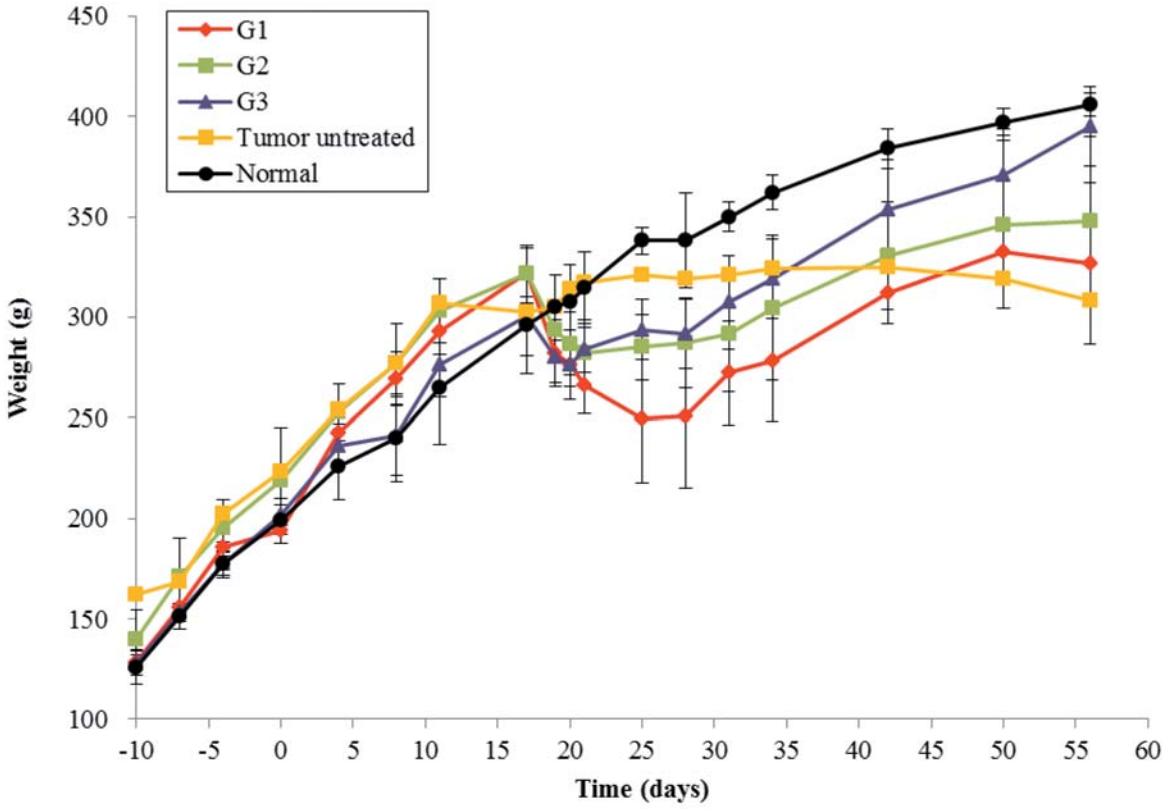

Figure 2. *Changes in body weight of liver tumor-bearing rats that had received various doses of Boron neutron capture therapy (BNCT) irradiation. BNCT-treated G1, G2 and G3 subgroups received physical doses of 11.18, 7.97, and 5.61 Gy, respectively. Body weights of the normal group and tumor-bearing untreated group, neither of which received boric acid, nor BNCT, were used for comparison (n=5-9).*

Table III. *Tumor reduction ratio[#] in rats as detected by ultrasound scanning before and after boron neutron capture therapy (BNCT) at 80 days.*

Treatment	Time relative to BNCT				
	1 Day before	11 Days after	30 Days after	60 Days after	80 Days after
Tumor comparison group	0	–0.55±0.2	–0.22±0.35	–0.48±0.17	–1.34±1.26
BNCT subgroups					
G1	0	0.64±0.07	0.94±0.01	0.94±0.02	0.95±0.02
G2	0	0.62±0.11	0.89±0.05	0.86±0.07	0.91±0.05
G3	0	0.48±0.42	0.77±0.06	0.83±0.08	0.87±0.04

G1, G2, and G3 subgroups received physical doses of 11.18 Gy, 7.97 Gy, and 5.61 Gy, respectively. Each subgroup comprised of seven rats. [#]The initial size of tumor (one day before BNCT treatment) was defined as 0; a negative value indicates an increase in tumor size, and a positive value indicates a decrease in tumor size. Data are means±SD (n=7).

normal tissues at acceptable levels. Two boron drugs, BPA and BSH, are currently in clinical use. The pharmacokinetic properties of BA as a boron drug for BNCT, including its transport, biodistribution, and accumulation, differ greatly from those of BPA and BSH. The permeability and mechanism of transport of BA across the plasma membrane has been studied, and the results suggested that BA transport involves simple passive diffusion through the lipid bilayer; the kinetics of BA uptake appeared to be linearly related to its concentration, and the plasma membrane permeability remained the same at different extracellular BA concentrations (16). Even though BA enters and leaves cells by simple diffusion, or and is not selectively accumulated, neither the relative

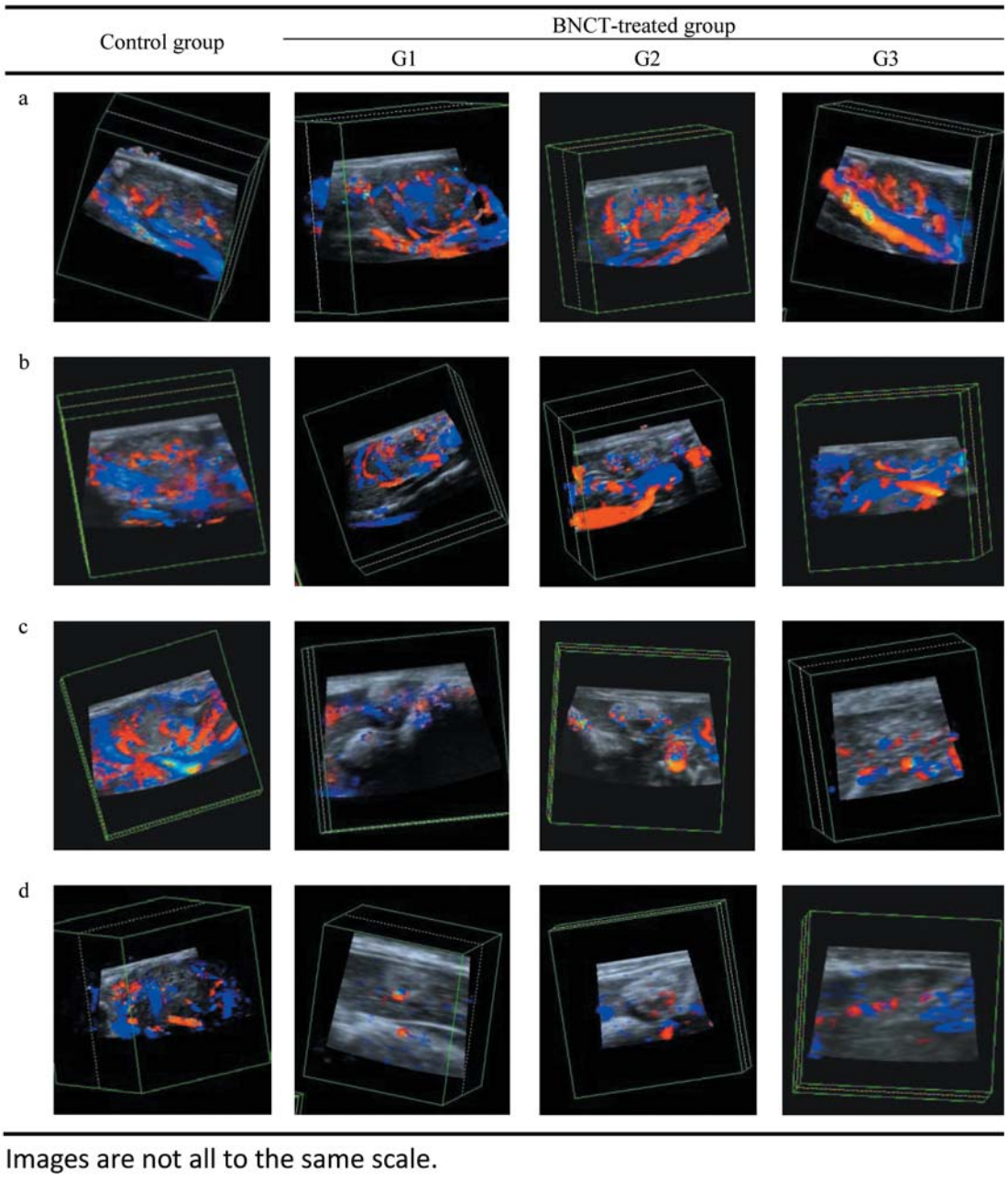

Figure 3. *Colour Doppler imaging reveals reduction of blood flow for liver tumor following boron neutron capture therapy (BNCT) treatment. Blood flow patterns one day before (a) and on the 8th (b), 11th (c) and 18th (d) day following BNCT. G1, G2 and G3 subgroups received 11.18, 7.97, and 5.61 Gy of physical doses under BNCT, respectively. Colour Doppler image of tumor from the tumor control group is shown for comparison.*

concentration of BA in tumor and normal tissue, nor its distribution in tumor, has been reported upon. A study of the permeability of BA across lipid bilayers suggested that the lipid composition of the plasma membrane affects total boron uptake (17). Differences in the content and proportions of fatty acids between cancer cell lines and their normal counterparts have been reported (31). This may be responsible for the fact that BA concentrations in tumor regions in this study are higher than those in normal tissues.

Sinusoids, a kind of capillary in HCC, are present around tumor cells. The morphology of the endothelial cells that constitute the sinusoids in HCC is distinct from that of sinusoidal endothelial cells in the normal liver (32, 33). The

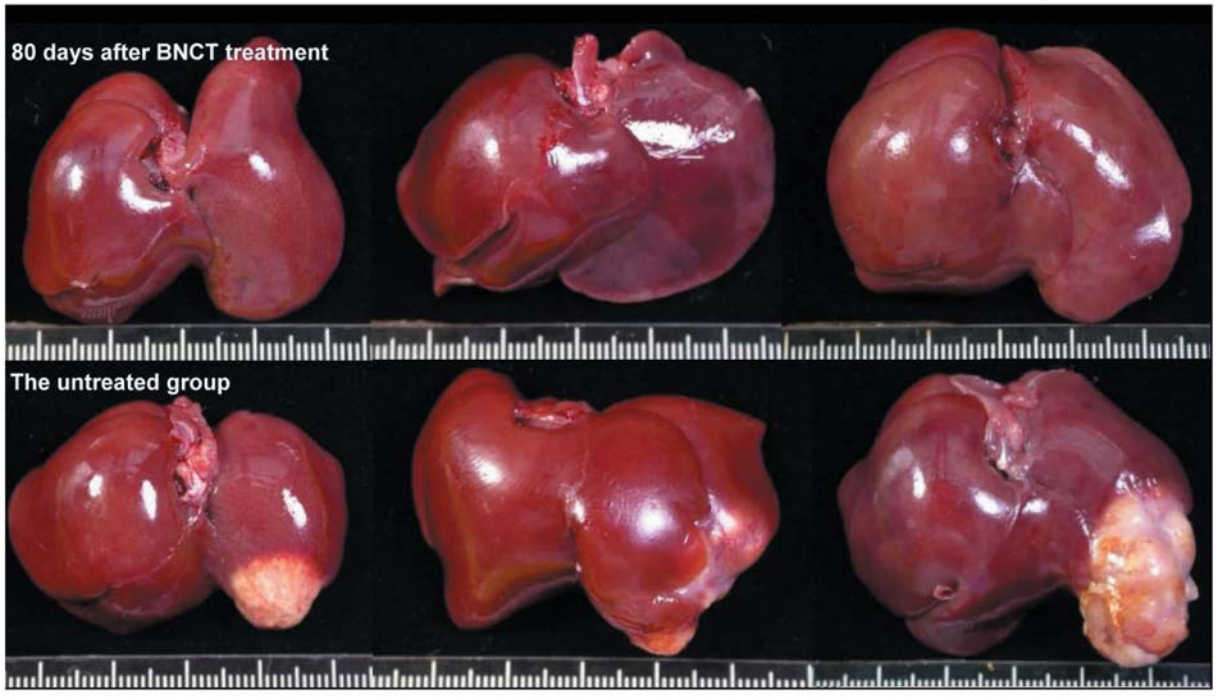

Figure 4. *Morphology of tumor-bearing liver on the 80th day after boron neutron capture therapy (BNCT). In the upper panel, the liver from BNCT-treated rats showed no tumor; in the lower panel, a large tumor was observed on the left liver lobe from rats of the tumor comparison group.*

necessity of transport across vessel walls or through interstitial spaces limits the uptake of BA, as it limits that of most currently used chemotherapeutic agents. Since the distribution and structure of vessels in the tumor differ from those in a normal tissue (34, 35), not surprisingly, the BA concentration in tumor herein exceeded that in normal liver tissue. Although the ratio of boron concentration in the tumor to that of normal liver, remained around 1.5 at 30 min after the administration of BA, an autoradiographic investigation revealed spots with a high density of alpha tracks adjacent to and within the tumors (data not shown). The tumor section was taken for histopathological investigation after it was used for neutron autoradiograpy. Spots with a high density of alpha tracks are regions of tumor vessels in which large amounts of BA are retained. BNCT is a binary treatment modality that relies upon the selective accumulation of a ^{10}B-containing compound in the critical region of the tumor, followed by irradiation of the tumor zone. The ^{10}B-containing tumor regions can be killed while the boron-poor normal tissue is spared. The retention of a high BA content in tumor vessels is assumed to be crucial to BA-mediated BNCT for hepatoma. Accordingly, the mechanisms by which BA-mediated BNCT completely eradicates tumors may not depend only on the fact that the boron concentration in tumor cells exceeds that in normal tissue. In this investigation, the selective reduction of tumor blood flow in BA-mediated BNCT was observed by colour Doppler imaging analysis. The findings revealed that the amount of blood flow in a tumor and its immediate surroundings gradually decreased until it was undetectable at 18 days following BNCT, indicating that disrupting tumor blood vessels is crucial in the BA-mediated BNCT for hepatoma. Similarly, endothelial cell damage has been reported to cause the breakdown of the blood alveolus barrier in the development of radiation-induced lung injury (36).

Radiation therapy has conventionally been of limited use in the treatment of liver tumors, mainly due to the low whole-organ tolerance of the liver to radiation: when the entire liver is irradiated, doses of 30 to 33 Gy carry a risk of about 5% for radiation-induced liver disease. Therefore, radiation therapy is unsuitable for treating liver cancer (37). However, the effect of BNCT on normal liver regeneration has been investigated in Wistar rats, and at the physical doses selected, have been found not to reduce the capacity of normal liver hepatocytes to regenerate (38). Additionally, the experimental results herein reveal that BNCT restored the liver function, reflected by GOT and GPT values, of tumor-bearing rats to a normal level. During the period of observation, after the 11th day post-BNCT, the groups that were treated with higher doses of BNCT (G1 and G2) exhibited a greater reduction in tumor size than the group that was treated with the lowest dose (G3). On the 80th day following BNCT, no significant difference existed among the

three groups, as the tumor disappeared in the tumor-bearing rats and the liver functions all of the groups recovered. Furthermore, although the BA in kidneys exceeded that in liver at the time of neutron irradiation (approximately 0.5 h following BA administration), the physical dose in the kidneys was less than that in the liver (data not shown). Therefore, the kidney function in rats, monitored by changes in BUN values, showed that the BNCT-treated groups exhibited no significant change upon BNCT treatment, whereas the tumor comparison group exhibited a clear increase in BUN. Kidney function seems to be unharmed in rats that are treated with BNCT that is mediated by BA. Therefore, BA-mediated BNCT can satisfy the requirements for internal targeting radiation therapy for liver cancer.

HCC is one of the most vascular solid tumors, and exhibits special vascular changes such as angiogenesis, arterialization and sinusoidal capillarization (39, 40). Blood vessels in tumors are recognized as being clinically important therapeutic targets. The abnormalities of tumor vessels enable them to be targeted without destroying normal vasculature (35). Based on the experimental results that are obtained herein, the rates of decrease in tumor size and blood flow was greatest during the first eight days post-BNCT, then declined. Accordingly, the damage to tumor vessels by BNCT may thus reduce the supply of blood to the tumor, resulting in radiation-induced necrosis of the tumor. Therefore, the therapeutic mechanism of BA-mediated BNCT differs from that of BNCT with BSH and BPA.

This study provides new information that can be exploited to improve *in situ* BNCT for HCC. A preliminary investigation of the biological effects of BA-mediated BNCT on normal liver tissue and adjacent organs in a hepatoma-bearing rat model has been conducted (20). The biologically effective dose depends on the relative biological effectiveness and compound biological effectiveness of the boron compound used and the dose components in each case (41, 42). There must be further evaluated for BA-mediated BNCT to calculate the biologically effective dose accurately. To improve the therapeutic efficacy, the detailed mechanisms and the fractionation treatment of BA-mediated BNCT are being investigated using second animal models with the ultimate goal of treating multifocal liver tumors (43). The treatment of HCC remains a serious challenge owing to the lack of an effective systemic therapy, and the disruption of blood vessels by BA-mediated BNCT may prove useful in novel treatment for HCC.

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