

Article

Preclinical Validation of the iBNCT001 Accelerator System for Boron Neutron Capture Therapy: In Vitro Efficacy, Beam Quality, and Radiation Safety Evaluation

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Abstract

Boron neutron capture therapy (BNCT) is a binary radiotherapy that is based on nuclear reactions between boron-10 and low-energy neutrons, which enables selective tumor cell killing. Although accelerator-based BNCT systems are increasingly being adopted, each platform requires independent biological validation. Here, we performed an in vitro pre-clinical evaluation of the linac-based iBNCT001 system employing a beryllium target in combination with the clinically approved boron drug SPM-011 (borofalan (¹⁰B)). Three complementary studies were conducted: (i) a cell-based BNCT efficacy study, (ii) a free-beam radiobiological evaluation, and (iii) a radiation leakage assessment using a human-phantom model. BNCT using iBNCT001 and SPM-011 induced clear boron concentration- and dose-dependent reductions in clonogenic survival across multiple tumor cell lines. Free-beam experiments determined a relative biological effectiveness (RBE) of 2.3 for the hydrogen dose component associated with high energy neutrons. In the phantom study, the maximum radiation leakage dose during head irradiation was 1.31 GyEq in the cervical region. Although this study is limited to in vitro biological assessments, the results provide non-clinical evidence supporting the efficacy, beam quality, and biological safety of iBNCT001 for future clinical BNCT applications.

Keywords: boron neutron capture therapy; accelerator-based BNCT; preclinical study; linear accelerator; beryllium target; SPM-011 (borofalan (¹⁰B))

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1. Introduction

Boron neutron capture therapy (BNCT) is a binary radiotherapy that exploits the nuclear reaction between boron-10 (^{10}B) and low-energy neutrons to generate high linear energy transfer (LET) particles, primarily α -particles and recoiling ^7Li nuclei, within boron-loaded cells. Because the ranges of these particles are limited to approximately one cell diameter (5–9 μm), the cytotoxic effect is spatially confined to cells containing sufficient ^{10}B , enabling highly selective tumor cell killing while sparing the surrounding normal tissues when tumor-selective boron accumulation is achieved [1,2]. Therefore, BNCT has been investigated for malignancies that are difficult to control with conventional radiotherapy, including high-grade gliomas, recurrent head and neck cancers, and malignant melanoma [2–4]. Early clinical BNCT relied on research nuclear reactors as neutron sources, which provided intense thermal or epithermal neutron beams suitable for inducing the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ reaction in the tumor. However, the use of reactor-based neutron sources has imposed substantial regulatory, logistical, and societal constraints, severely limiting the availability and scalability of BNCT in routine clinical practice [5,6].

Accelerator-based neutron sources have been developed over the past two decades to overcome the limitations associated with reactor-based BNCT [6–8]. These systems generate epithermal neutrons through nuclear reactions induced by charged-particle beams incident on suitable target materials, thereby enabling compact, hospital-based BNCT facilities. Several accelerator platforms have been reported, including cyclotron-based systems employing beryllium targets and linac-based systems employing lithium targets [7,9–12]. In Japan, the cyclotron-based epithermal neutron source (C-BENS) using a beryllium target was one of the first accelerator-based BNCT systems to demonstrate both pre-clinical feasibility and clinical efficacy [3,13–15]. Recently, linac-based systems, such as CICS-1, using a lithium target have been developed and introduced into clinical trials [12,16–18]. Although these systems share the common goal of generating epithermal neutron beams suitable for BNCT, differences in the accelerator type, target material, beam-shaping assembly, and neutron energy spectrum can substantially influence the physical dose components and biological effectiveness. Consequently, biological effects cannot be extrapolated solely from physical dosimetry, and each accelerator-based BNCT system requires independent biological validation prior to clinical application [9,19,20].

The choice of target material for neutron production is a critical determinant of the neutron yield, energy spectrum, and associated gamma-ray contamination [7,20]. Beryllium and lithium are the most commonly used target materials in accelerator-based BNCT systems; however, they differ substantially in nuclear reaction pathways and resulting neutron spectra [7,12]. Beryllium targets typically generate neutrons via (p,n) reactions with a relatively high neutron yield and a broad energy distribution, whereas lithium targets produce neutrons via the near-threshold $^7\text{Li}(\text{p},\text{n})^7\text{Be}$ reaction, resulting in a narrower energy spectrum. These differences may influence not only the physical dose distributions but also the biological effectiveness, underscoring the necessity of system-specific biological evaluation [9,10,20]. Despite the increasing interest in compact linac-based BNCT systems, linac-based systems employing beryllium targets remain insufficiently characterized from a biological perspective, particularly when compared with cyclotron-based Be systems or linac-based Li systems. Consequently, biological response data obtained from lithium-target BNCT systems or cyclotron-based beryllium systems cannot be directly extrapolated to beryllium-target linac-based platforms. In particular, an integrated in vitro biological validation addressing therapeutic efficacy, intrinsic beam quality, and biological safety has not been comprehensively reported for these systems. This lack of consolidated biological evidence represents a critical gap in the translational pathway of beryllium-target linac-based BNCT. SPM-011 (borofaran (^{10}B)) is currently the only boron-containing drug used in clinical boron neutron capture therapy (BNCT) in Japan. The active

pharmaceutical ingredient of SPM-011 is ^{10}B -borono-L-phenylalanine (^{10}BPA), which is taken up into tumor cells primarily via amino acid transport mechanisms, particularly L-type amino acid transporter 1 (LAT1), which is overexpressed in many malignant tumors. This transport selectivity enables the preferential accumulation of ^{10}B in tumor tissues [21,22]. Because SPM-011 is already used in clinical BNCT settings, preclinical evaluations of BNCT systems should be conducted using SPM-011 under conditions relevant to clinical practice. This approach ensures the translational relevance and regulatory acceptability of preclinical data supporting subsequent clinical trials [23,24].

The iBNCT001 system is a linac-based accelerator BNCT platform developed by the University of Tsukuba in collaboration with the High-Energy Accelerator Research Organization (KEK). In contrast to previously reported linac-based BNCT systems employing lithium targets, iBNCT001 adopts a beryllium target for neutron production, based on a design concept that prioritizes a high neutron yield, operational stability, and reduced radiation activation [10,25]. The iBNCT001 neutron source consists of a high-current proton linac system comprising an ion source, radio-frequency quadrupole (RFQ), drift tube linac (DTL), beam transport system, beryllium target, and beam shaping assembly (BSA), followed by an irradiation geometry optimized for biological experiments (Figure 1). Based on extensive conceptual design studies, the incident proton energy was set to 8 MeV to balance the efficient neutron production with radiation safety. At this energy, neutrons emitted from the $\text{Be}(p,n)$ reaction have energies below approximately 6.1 MeV, which effectively suppresses high-threshold activation reactions in structural and shielding materials, thereby reducing the long-lived residual radioactivity within the system [26].

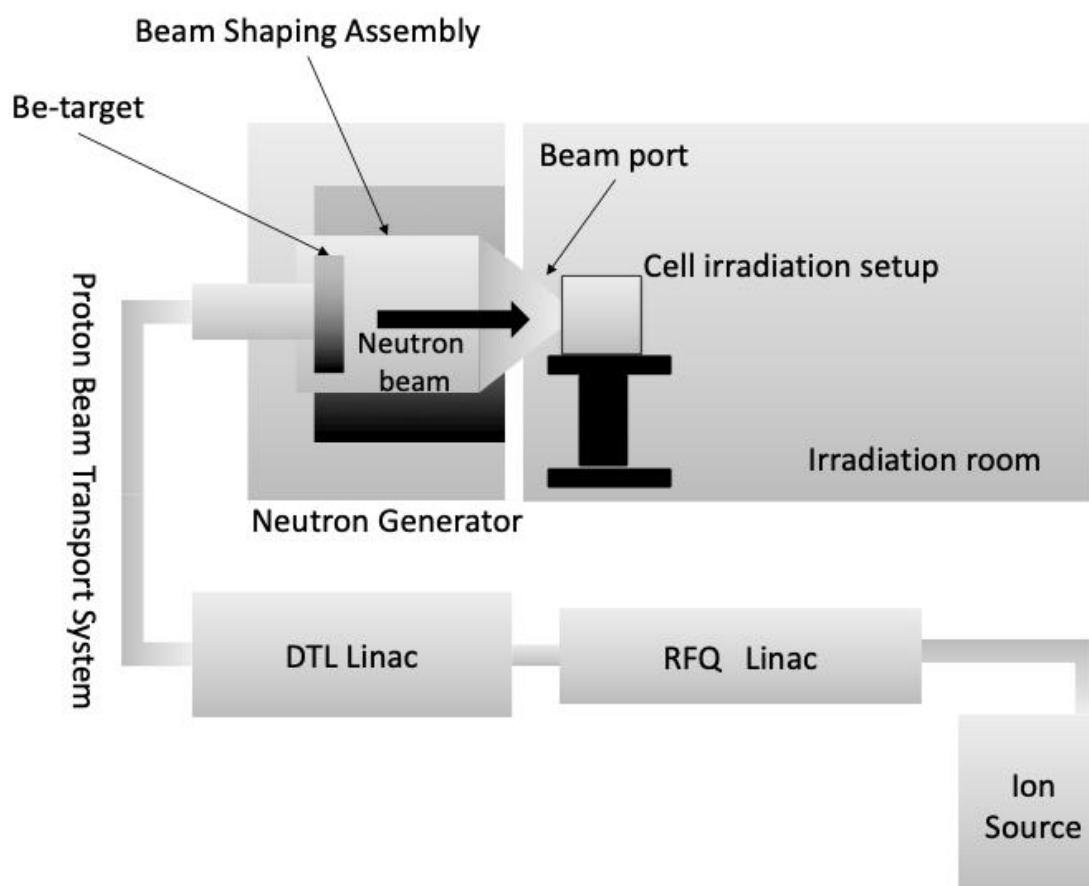


Figure 1. Schematic overview of the linac-based iBNCT001 neutron source and irradiation geometry used in this study. The system comprises an ion source, radio-frequency quadrupole (RFQ), drift tube linac (DTL), beryllium neutron production target, and beam-shaping assembly. Neutrons generated at the beryllium target were moderated and shaped before delivery to the cell irradiation

setup used in the present in vitro experiments. This schematic illustrates the overall system configuration and experimental geometry and is not drawn to scale.

To generate a sufficient neutron flux for BNCT at this relatively low proton energy, iBNCT001 employs a high-current linear accelerator capable of delivering an average proton beam current exceeding 5 mA, with a maximum current of up to 10 mA, corresponding to a proton beam power of 40–80 kW on a beryllium target. This combination of low proton energy and high beam current is a distinctive feature of iBNCT001, enabling efficient neutron production while maintaining low system activation and improving operational safety for routine clinical use. Given this unique accelerator configuration and low-activation design strategy, comprehensive preclinical validation is required to establish biological effectiveness, neutron beam quality, and radiation safety before clinical application. In Japan, regulatory frameworks emphasize robust nonclinical evidence supporting both efficacy and safety before the initiation of clinical trials [20,27]. Therefore, independent biological and dosimetric validation of the iBNCT001 system is essential to support its translational use as a clinically applicable accelerator-based BNCT neutron source.

From a clinical and practical perspective, accelerator-based BNCT systems fundamentally differ from conventional reactor-based BNCT facilities. Reactor-based BNCT relies on nuclear research reactors, which impose substantial limitations in terms of installation, regulatory burden, availability, and integration into hospital settings. In contrast, accelerator-based BNCT systems can be installed within medical institutions, operate under hospital-compatible safety frameworks, and provide on-demand neutron production without using a nuclear reactor. These features improve the accessibility, scalability, and long-term sustainability of BNCT while facilitating its integration with modern radiotherapy workflows. Consequently, accelerator-based platforms are increasingly regarded as the preferred approach for clinical BNCT implementation [5,6,8].

The objective of the present study was to perform integrated preclinical in vitro validation of the iBNCT001 accelerator system using the clinically approved boron compound SPM-011. Three complementary preclinical studies were conducted: (i) a cell-based BNCT efficacy study to quantify the biological equivalent dose (D_{10}), (ii) a free-beam biological evaluation to assess the intrinsic neutron beam quality and relative biological effectiveness (RBE), and (iii) a radiation leakage dose assessment using a human phantom to evaluate system safety. Through the integration of these studies, we aimed to establish a comprehensive biological and safety profile of the iBNCT001 system as a foundation for future clinical trials of BNCT.

In the context of BNCT system development and regulatory science in Japan, rigorously documented in vitro biological studies conducted under predefined experimental protocols constitute an essential component of preclinical validation prior to the first-in-human application. Accordingly, the present study focuses on the in vitro preclinical biological validation of the iBNCT001 accelerator system, while acknowledging that in vivo validation represents a subsequent and necessary step.

2. Materials and Methods

2.1. Overview of Preclinical Studies

This study comprised three independent, but complementary preclinical in vitro investigations conducted using a linac-based accelerator BNCT system, iBNCT001 (custom-built BNCT system jointly developed by the University of Tsukuba and KEK, Tsukuba, Japan). A cell-based BNCT efficacy study was performed to quantify tumor cell killing and determine the biologically equivalent dose. A free-beam biological evaluation was conducted to assess the intrinsic biological effectiveness of neutron beams independent of boron administration. In addition, a radiation leakage dose assessment using a human

phantom was performed to evaluate radiation safety for future clinical implementation. All studies were conducted in accordance with the finalized study protocols, including approved amendments and correction documents, and were subjected to quality assurance procedures. Each in vitro experiment was conducted according to a predefined experimental protocol.

For all clonogenic survival assays, each experimental condition consisted of three independent biological replicates ($n = 3$), and each replicate was derived from a separately prepared cell culture and independently irradiated sample. Survival fractions were calculated from colony counts obtained after a defined incubation period, and survival curves were constructed using the experimentally measured absorbed doses. No data-driven training, test splitting, or model optimization procedures were applied because this study was based on hypothesis-driven experimental radiobiology rather than data-driven prediction or machine learning approaches.

2.2. Cell-Based Preclinical Efficacy Study of BNCT

2.2.1. Study Design and Objective (In Vitro BNCT Efficacy)

This study aimed to evaluate the in vitro cytotoxic efficacy of BNCT using the iBNCT001 accelerator system in combination with a clinically approved boron compound, SPM-011. SPM-011 (borofaran (^{10}B); Lot No. 20S01B) was supplied free of charge by Stella Pharma Co. Ltd, Osaka, Japan. Tumor cell killing was quantified using clonogenic survival assays, and biological effectiveness was evaluated by determining the absorbed dose required to reduce the surviving fraction to 50% (D_{50}) and 10% (D_{10}). This study was designed to provide preclinical biological evidence supporting the therapeutic potential of the iBNCT001 system for clinical use.

2.2.2. Cell Lines

The following human cancer cell lines were used: SAS (human oral squamous cell carcinoma), T98G and A172 (human glioblastoma), and COLO679 and G-361 (human malignant melanoma). All cell lines were obtained from the RIKEN Bioresource Research Center (RIKEN BRC, Tsukuba, Japan), a publicly funded and internationally recognized cell bank that ensures cell line authentication and quality control. The RIKEN Cell Bank (RCB) numbers were as follows: SAS (RCB1974), T98G (RCB1954), A172 (RCB2530), COLO679 (RCB0989), and G-361 (RCB0991). These cell lines were selected based on their clinical relevance to malignancies for which BNCT has been investigated or clinically applied, including head and neck cancer, high-grade glioma, and malignant melanoma.

2.2.3. Cell Culture Conditions

Cells were cultured in Eagle's minimum essential medium (EMEM; 051-07615, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Lot No. 2404079, Thermo Fisher Scientific K.K., Tokyo, Japan) and antibiotics (100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin; Merck KGaA, Darmstadt, Germany). Cell cultures were maintained at 37 °C in a humidified incubator with 5% CO₂ dioxide. Cells were routinely passaged to maintain exponential growth, and experiments were conducted using cells within a defined passage range, as specified in the study protocol.

2.2.4. SPM-011 Treatment Conditions

SPM-011 was used as a boron delivery agent for BNCT. Cells were treated with SPM-011 at four different ^{10}B concentrations (0, 10, 25, and 40 ppm) to evaluate concentration-dependent biological effects. Working solutions were prepared by diluting the SPM-011

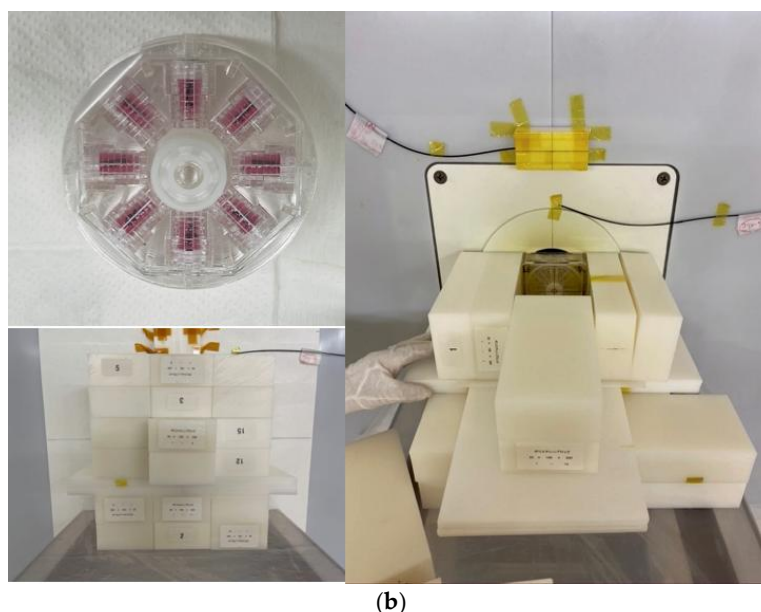


Figure 2. In vitro BNCT irradiation geometry used for the cell-based efficacy study with iBNCT001 system. **(a)** Schematic illustration of the irradiation setup. Cell suspensions (0.8 mL) were loaded into cryogenic tubes and fixed in a dedicated irradiation holder surrounded by water-equivalent materials (acrylic and polyethylene). A 20 mm thick water-equivalent layer was placed upstream of the samples to ensure appropriate neutron thermalization before interaction with the cells. The grey dashed line indicates the boundary separating the top view (view from above the beam) and the front view (view from the beam direction). The yellow circle represents the effective neutron beam aperture at the beam exit. **(b)** Photograph of the actual irradiation setup showing the sample holder and tube fixation during neutron irradiation.

Irradiation was conducted at room temperature, and a single irradiation was applied to each experimental group. The nominal irradiation times were set to 5, 10, 20, 30, and 60 min, corresponding to proton charges of 600, 1200, 2400, 3600, and 7200 mC, respectively, assuming an average proton beam current of 2.0 mA. Owing to minor fluctuations in the beam current during accelerator operation, the actual irradiation times ranged from 5.20–5.45 min, 10.15–11.10 min, 19.93–21.62 min, 31.23–32.92 min, and 59.90–64.67 min. These irradiation conditions were selected to ensure that for each boron concentration, at least four experimentally measured data points were obtained within each survival curve, enabling a robust evaluation of the relationship between neutron fluence or absorbed dose and clonogenic survival. Following irradiation, the cell samples were immediately transported to the biological laboratory, and clonogenic survival assays were performed without delay. All irradiation parameters, including boron concentrations and proton charge settings, were predefined prior to the experiments, according to the study protocol. The irradiation times and corresponding proton charges were selected to ensure that at least four experimentally measured data points were obtained within the survival curve for each boron concentration. No post hoc parameter tuning or data-driven optimization was performed.

2.2.6. Dosimetry and Absorbed Dose Calculation

The absorbed doses used for cell survival analysis were determined based on a combination of Monte Carlo simulations and experimental dosimetry. Dose calculations were performed using the Particle and Heavy Ion Transport code System (PHITS, version 2.52) Monte Carlo code by the medical physics group to estimate the absorbed dose components at the cell irradiation position under the defined irradiation geometry. The

simulation model incorporated the neutron energy spectrum, irradiation geometry, and material composition of the cell irradiation setup, as previously described for the iBNCT001 system [10]. To ensure consistency with the actual irradiation conditions, the simulated absorbed dose rates were normalized using the experimentally measured neutron fluence obtained from gold wire activation analysis and gamma-ray dose measured using radiophotoluminescence glass dosimeters (RPLDs; GD-301, Chiyoda Technol Corporation, Tokyo, Japan) placed within the cell irradiation holder. Based on this normalization, the absorbed dose rates per unit proton beam current (Gy/s/mA) were derived for each boron concentration condition. For the efficacy study, absorbed dose rates of 8.16×10^{-4} , 1.15×10^{-3} , 1.65×10^{-3} , and 2.15×10^{-3} Gy/s/mA were used for boron concentrations of 0, 10, 25, and 40 ppm, respectively. The absorbed dose delivered in each experiment was calculated by multiplying the dose rate (Gy/s/mA) by the average proton beam current (mA) and irradiation time (s) recorded for each irradiation. The total absorbed dose was defined as the sum of the dose components attributable to the boron reaction, nitrogen capture, hydrogen recoil, and gamma-ray contributions, and was used as the dose metric for subsequent biological evaluations. This dosimetric evaluation and dose calculation procedure was conducted as a hypothesis-driven experimental analysis rather than a data-driven or predictive modeling approach. No data-driven training, test splitting, or model-optimization procedures were applied. Absorbed dose calculations were deterministically derived from Monte Carlo simulations normalized by experimental dosimetry, and the resulting dose values were directly used for biological endpoint evaluation in clonogenic survival assays. In these simulations, the absorbed dose components arising from the boron neutron capture reaction, nitrogen capture, hydrogen recoil, and accompanying gamma rays were separately tallied using standard PHITS particle- and reaction-based scoring functions. The nuclear reaction cross-section data implemented in the default PHITS nuclear data libraries were used without modification. No additional assumptions or empirical correction factors were introduced beyond the experimentally based normalization described previously.

2.2.7. Quality Assurance and Commissioning of Neutron Irradiation

For quality assurance and commissioning of neutron dose delivery in the efficacy study, neutron fluence verification using gold wire activation analysis was performed during the study period. Water phantom irradiations were conducted prior to the first cell irradiation and after the completion of the final cell irradiation to confirm the baseline neutron fluence. In addition, on each cell irradiation day, the gold wires were irradiated at two predefined positions within the irradiation geometry. The induced radioactivity of the gold wires was measured using a Ge semiconductor detector (GEM20P4-70, ORTEC, Oak Ridge, TN, USA). Based on the gold wire mass and delivered proton charge, the specific radioactivity per unit mass and per unit charge (Bq/mg/mC) was calculated as follows: The measured values were compared with the reference mean specific radioactivity per unit charge obtained from 14–15 calibration irradiations performed under the same irradiation geometry before the present study was initiated. All measurements were within $\pm 5\%$ of the reference values, confirming the validity and consistency of the neutron dose delivery during the study period.

2.2.8. Clonogenic Survival Assay and Determination of Biological Equivalent Dose

After neutron irradiation, the cryogenic tubes were immediately transported to a biological laboratory. The tubes were centrifuged to pellet the cells, and the supernatant was carefully removed (as it could be activated by neutron exposure). The cell pellet was resuspended in 0.8 mL of fresh culture medium and transferred to a round-bottom tube for further processing. Cell concentration was measured three times using an automated cell

counter, and the mean value was used to determine the appropriate serial dilutions and seeding volumes. Cells were then replated into 60 mm dishes (three dishes per condition) at densities selected to yield a countable number of colonies. Cells were incubated for colony formation for 7–18 d, depending on the cell line and growth rate. The colonies were fixed with 10% formalin for 10 min at room temperature and stained with 1% methylene blue for 10 min. Colonies containing ≥ 50 cells were considered surviving colonies. The plating efficiency (PE) was calculated from the unirradiated control as follows:

$$PE = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \quad (1)$$

For each irradiated sample, the surviving fraction (SF) was calculated using PE-normalized colony counts as follows:

$$SF = \frac{\text{colonies counted}}{\text{cells seeded} \times PE} \quad (2)$$

Survival curves were generated by plotting the SF as a function of the absorbed dose on a semi-logarithmic scale. Curve fitting was performed primarily using the linear–quadratic (LQ) model, expressed as

$$SF = \exp(-\alpha D - \beta D^2) \quad (3)$$

where *SF* represents the surviving fraction, and *D* is the absorbed dose. In cases where a pronounced tailing effect was observed at higher dose ranges, curve fitting was performed using a linear (L) model, expressed as

$$SF = \exp(-\alpha D) \quad (4)$$

in accordance with the study's protocol. The fitting procedure yielded the radiobiological parameters α and/or β , which were subsequently used to derive the biologically effective doses. Based on the fitted survival curves, the absorbed doses corresponding to surviving fractions of 0.5 (D_{50}) and 0.1 (D_{10}) were calculated for each cell line and experimental condition. These dose metrics were used as indices of biological effectiveness for the comparative evaluation of BNCT efficacy. Statistical analyses were conducted according to the predefined procedures.

2.3. Evaluation of Biological Effects Under Free Beam Conditions

2.3.1. Study Design and Objective (Free-Beam RBE Evaluation)

This study evaluated the biological effectiveness and beam quality of the neutron beam generated by the iBNCT001 accelerator system in free air. Cellular survival following neutron irradiation under free-beam conditions was compared with that following reference X-ray irradiation to determine the RBE based on clonogenic survival.

2.3.2. Cell Lines and Culture Conditions

The following cell lines were used: CHO-K1 (Chinese hamster ovary), SAS, T98G, A172, COLO679, and G-361. CHO-K1 cells were obtained from the JCRB Cell Bank (JCRB9018), and all human cancer cell lines were obtained from the RIKEN Bioresource Research Center, as described in Section 2.2.2. The cell culture conditions, media composition, incubation environment, passaging procedures, and preparation of exponentially growing cells were identical to those described in Section 2.2.3.

2.3.3. Preparation of Irradiation Samples

Cells were seeded and cultured for at least 24 h before irradiation to ensure logarithmic growth at the time of exposure. For irradiation, the cells were washed once with PBS (–), dissociated into a single-cell suspension using trypsin–EDTA, and resuspended in

fresh culture medium without any boron compound. The cell suspension was adjusted to a predefined concentration and aliquoted (0.8 mL) into cryogenic tubes for neutron irradiation, as described in Section 2.2.4. Subsequent clonogenic survival processing (sample recovery after irradiation, cell counting, dilution and replating, colony fixation/staining, colony counting, and calculation of surviving fractions) was performed according to the workflow described in Section 2.2.8.

2.3.4. Neutron Irradiation Under Free-Beam Conditions

For free-beam irradiation experiments, cell suspensions were prepared as described above and aliquoted into cryogenic tubes (0.8 mL each). The tubes were fixed in a dedicated free-beam irradiation case fabricated from low-density Styrofoam (4100-styib900-15-2; thickness, 15 mm; width, 300 mm; length, 300 mm; In The Box, Package Art Co., Ltd., Yanai City, Japan) providing sufficient mechanical stability while minimizing neutron scattering and attenuation. This configuration ensured that only air, the tube wall, and the culture medium were present between the neutron beam exit and the cell samples, thereby enabling irradiation under true free-beam conditions. The fixation geometry and relative positioning of the samples with respect to the beam port are illustrated (Figure 3a (scheme) and Figure 3b (photograph)).

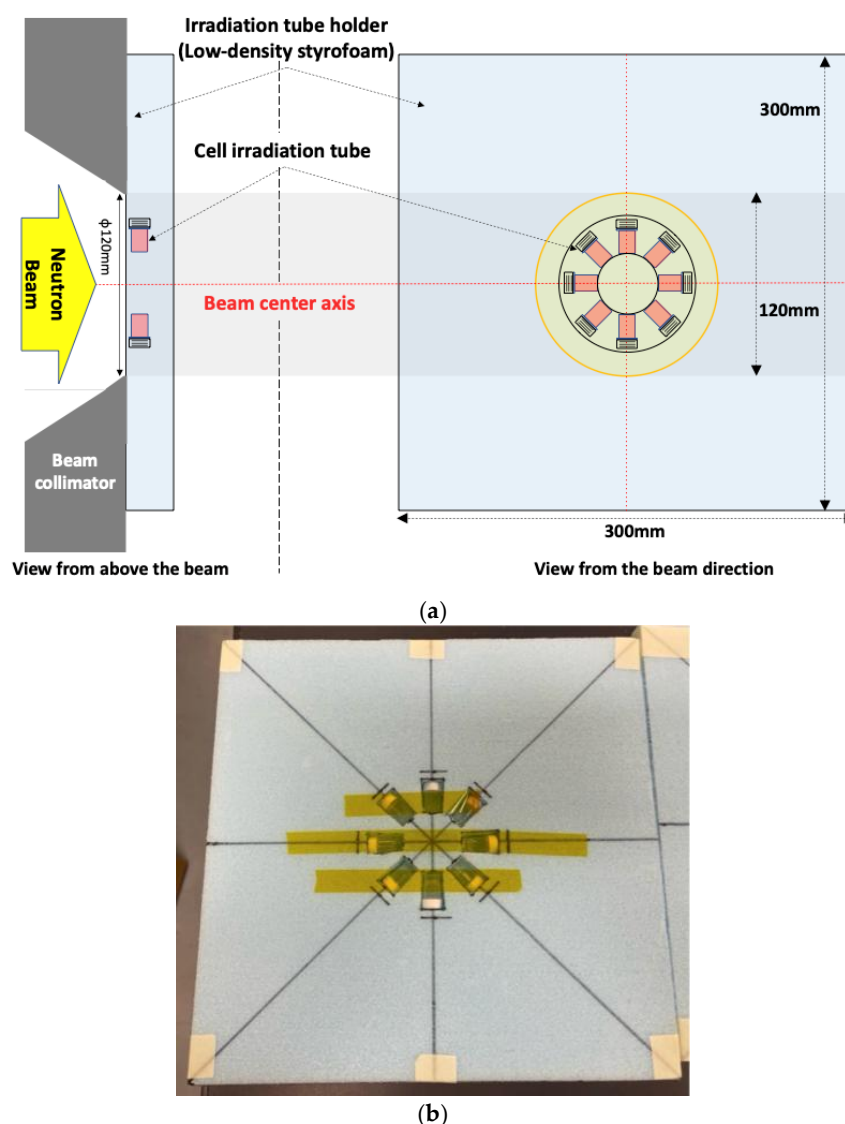


Figure 3. Free-beam neutron irradiation geometry for evaluating the intrinsic biological effectiveness of neutron beams. (a) Schematic overview of the free-beam irradiation configuration. Cell

suspension tubes were fixed in a custom-made holder fabricated from low-density Styrofoam to minimize neutron scattering and attenuation, allowing irradiation under actual free-beam conditions. The grey dashed line indicates the boundary separating the top view (view from above the beam) and the front view (view from the beam direction). The yellow circle represents the effective neutron beam aperture at the beam exit. **(b)** Photograph of the free-beam irradiation case positioned at the beam port, illustrating the relative placement of the sample tubes with respect to the neutron beam exit.

Neutron irradiation was performed at room temperature with a single irradiation per experimental group. The nominal irradiation times were set to 30, 60, and 90 min, corresponding to proton charges of 3600, 7200, and 10,800 mC, respectively, assuming an average proton beam current of 2.0 mA. The actual irradiation times ranged from 31.23–32.17 min for the 30 min condition, 59.18–62.85 min for the 60 min condition, and 92.57–94.38 min for the 90 min condition, reflecting minor variations in beam current during operation. These irradiation conditions were selected to obtain at least four data points, including the unirradiated control, for each survival curve.

For the free-beam irradiation experiments, the absorbed doses were calculated using the same Monte Carlo-based approach described above. PHITS simulations were performed for the free-beam irradiation geometry to estimate the absorbed dose rate at the cell positions. The simulated results were normalized using gold wire activation measurements and RPLDs-based gamma-ray dose measurements obtained under identical free-beam conditions. Based on this procedure, an absorbed dose rate of 3.79×10^{-4} Gy/s/mA was derived for the free beam configuration. The absorbed doses used for the survival curve analysis were calculated from the experimentally recorded proton beam current and irradiation time for each experiment.

2.3.5. Quality Assurance and Commissioning of Neutron Irradiation

For the free-beam irradiation experiments, quality assurance procedures were performed on each irradiation day, prior to cell exposure. The neutron fluence was verified using gold wire activation analysis in a water phantom, and cell irradiation was initiated only after confirming that the deviation from the reference neutron fluence was within $\pm 5\%$. After completion of the final cell irradiation experiment, an additional water phantom irradiation was performed to confirm the neutron fluence stability. The measured neutron fluence remained within $\pm 5\%$ of the reference values, and the maximum thermal neutron flux exceeded 1.0×10^9 n/cm²/s, ensuring a stable and reproducible beam output throughout the study.

2.3.6. Reference X-Ray Irradiation

Reference X-ray irradiation was performed using a predefined irradiation geometry (Figure 4) designed to match, as closely as practical, the sample fixation and surrounding scattering conditions used for neutron irradiation. X-ray irradiation was conducted at 130 kV and 5 mA with a 0.5 mm aluminum filter using a Cabinet X-ray Irradiator System (RX-650, Faxitron X-ray LLC, Tucson, AZ, USA). The X-ray dose rate at the sample position was determined prior to the study by inserting calibrated thermoluminescent dosimeters (TLDs; GD-352M, AGC Techno Glass Co., Ltd., Shizuoka, Japan) into the center of a sample tube filled with 0.8 mL of agar medium under the same geometry used for cell irradiation. Based on the measurements of multiple TLD elements, the mean dose rate was set to 0.47 ± 0.02 Gy/min. Absorbed doses of 0, 2, 4, 6, and 8 Gy were delivered as single exposures, corresponding to irradiation times of 4 min 16 s, 8 min 33 s, 12 min 49 s, and 17 min 05 s for 2, 4, 6, and 8 Gy, respectively. These dose levels were predefined to obtain at least four experimentally measured data points within each survival curve for a robust

dose–response fitting. Using a consistent irradiation geometry for neutron and X-ray exposure was intended to minimize variations in the scatter and secondary radiation components, thereby enabling accurate RBE determination.

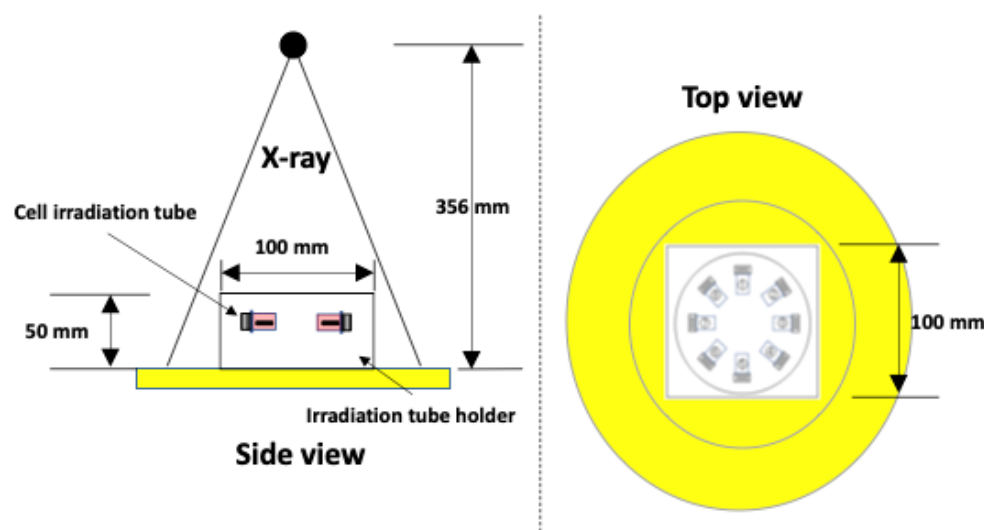


Figure 4. Reference X-ray irradiation geometry used for clonogenic survival and RBE determination. Schematic representation of the X-ray irradiation setup designed to match, as closely as practical, the sample fixation and surrounding scattering conditions used for neutron irradiation. The gray dashed line indicates the separation between the side view and the top view of the irradiation geometry. The yellow-colored area represents a flat irradiation support plate on which the irradiation tube holder containing the sample tubes was placed during X-ray exposure; in the top view, the outer circle denotes the boundary of the support plate, while the inner circle indicates the effective X-ray irradiation field. X-ray irradiation was performed using a cabinet X-ray irradiator at 130 kV and 5 mA with a 0.5 mm aluminum filter. The dose rate at the sample position was determined using thermoluminescent dosimeters placed at the center of an agar-filled sample tube with an identical geometry.

2.3.7. RBE Determination

Clonogenic survival assays, survival curve fitting, and derivation of D_{10} values were performed as described in Section 2.2.7. The RBE of fast neutrons was evaluated based on the absorbed dose required to reduce the surviving fraction to 10%. Because the neutron beam generated by the iBNCT001 system contains a non-negligible gamma-ray component, the contribution of gamma rays to cell death was explicitly separated prior to the RBE calculation, following the approach reported in previous studies [28,29]. Briefly, the gamma-ray dose fraction under free-beam irradiation geometry was quantified experimentally using RPLDs. Based on five independent measurements using 13 dosimeters, the gamma-ray contribution was determined to be 19.18% of the total absorbed dose. The RBE was calculated using the following equation:

$$\text{RBE} = \frac{D_{10,X} - D_{10,N\gamma}}{D_{10,N} - D_{10,N\gamma}} \quad (5)$$

where $D_{10,X}$ is the absorbed X-ray dose corresponding to 10% survival, $D_{10,N}$ is the total absorbed dose under neutron irradiation corresponding to 10% survival, and $D_{10,N\gamma}$ is the gamma-ray dose component included in $D_{10,N}$. By subtracting the gamma-ray contribution from both $D_{10,X}$ and $D_{10,N}$, the influence of photon contamination on the evaluated neutron biological effectiveness was minimized. Survival curves were fitted using the LQ model for X-ray irradiation. For neutron irradiation, survival curves were fitted using a linear model after applying the gamma-ray subtraction procedure, consistent with the high-

LET-like characteristics of fast neutron irradiation. The resulting neutron-only D_{10} values were used to derive the RBE values for each cell line.

2.4. Radiation Leakage Dose Assessment Using a Human Phantom

2.4.1. Study Design and Objective (Radiation Leakage Dose Assessment)

This study was conducted to evaluate out-of-field leakage radiation doses associated with neutron irradiation using the iBNCT001 accelerator system under clinically relevant BNCT conditions. To simulate patient exposure during BNCT, a human whole-body phantom was employed, and the biological effects induced by leakage radiation were quantified using a micronucleus (MN) formation assay. Leakage doses at multiple anatomical locations were estimated by converting the observed MN frequencies to X-ray-equivalent doses based on a reference X-ray dose–response relationship.

2.4.2. Irradiation System and Boron Compound

The neutron irradiation system used in this study was the iBNCT001 linac-based accelerator system equipped with a beryllium target, as described in Sections 2.2.5 and 2.3.4. System stability was confirmed throughout the study period by verifying that the maximum thermal neutron flux in a water phantom exceeded 1.0×10^9 n/cm²/s after completion of the final irradiation (measured value: 1.169×10^9 n/cm²/s). SPM-011 was used as the boron compound. The stock formulation contained ¹⁰B at a concentration of 1440.9 ppm and was diluted with culture medium to achieve a final ¹⁰B concentration of 25 ppm for cell treatment. Storage conditions, dilution procedures, and handling protocols were identical to those described in Section 2.2.4.

2.4.3. Cell Line and Culture Conditions

Chinese hamster ovary (CHO-K1) cells were used for cytogenetic evaluation. CHO-K1 cells were obtained from the JCRB of Research Bioresources Cell Bank (JCRB9018). The cell culture conditions, including medium composition, incubation environment, passaging procedures, and preparation of logarithmically growing cells, were identical to those described in Section 2.2.3.

2.4.4. Preparation of Cell Samples and Boron Treatment

Cells were cultured for at least 24 h prior to irradiation to ensure exponential growth. After removal of the culture medium, cells were treated with SPM-011-containing medium at a ¹⁰B concentration of 25 ppm for 1 h. Following exposure, the treatment solution was aspirated, cells were washed with PBS (–), and dissociated into a single-cell suspension using trypsin–EDTA. Cells were subsequently collected in fresh culture medium containing the same ¹⁰B concentration (25 ppm) to maintain boron availability during neutron irradiation. This procedure ensured uniform boron exposure and was designed to represent a conservative condition corresponding to continuous boron administration in clinical BNCT.

2.4.5. Neutron Irradiation Using a Human Phantom Model

To evaluate out-of-field leakage radiation doses at various body regions during BNCT, a human whole-body phantom was employed to approximate the patient anatomy under clinically realistic irradiation conditions. Cell suspension samples were attached to predefined anatomical locations on the phantom surface, enabling the direct biological assessment of leakage radiation at each site. This irradiation method was selected to simulate patient exposure during head BNCT and to provide a conservative evaluation of whole-body leakage doses in a worst-case clinical scenario. Neutron irradiation was

performed using the iBNCT001 linac-based accelerator system under conditions corresponding to a conservative maximum assumed clinical tumor dose of 10 GyEq for malignant gliomas [30]. This reference condition was defined based on previous physical dose measurements and Monte Carlo simulations conducted for an ongoing clinical BNCT trial. Based on these evaluations, a proton charge of 3625 mC corresponded to the prescribed dose level. This charge is equivalent to irradiation at an average proton beam current of 2.0 mA for approximately 30.2 min. In the actual experiments, neutron irradiation was performed at room temperature, with measured irradiation times ranging from 31.2 to 31.7 min, reflecting minor fluctuations in the beam current during operation.

A human whole-body phantom fabricated from an acrylic water-equivalent material was used to simulate patient exposure [31]. The phantom represented an adult human body (height, 168.0 cm; weight, 58.1 kg) and consisted of block-shaped compartments corresponding to anatomical regions, including the head, neck, chest, abdomen, inguinal region, thigh, lower leg, and ankle. To minimize the distance between the irradiation port and the phantom, all regions except the left arm were included in the irradiation setup. Cell suspensions (0.8 mL) were aliquoted into FastGene 0.5 mL cryogenic tubes (FG-CRY-In-05S; Nippon Genetics Co., Ltd., Tokyo, Japan) and fixed at predefined anatomical locations on the phantom using Kapton tape, according to a predefined fixation diagram (Figure 5a (scheme) and Figure 5b (photograph)).

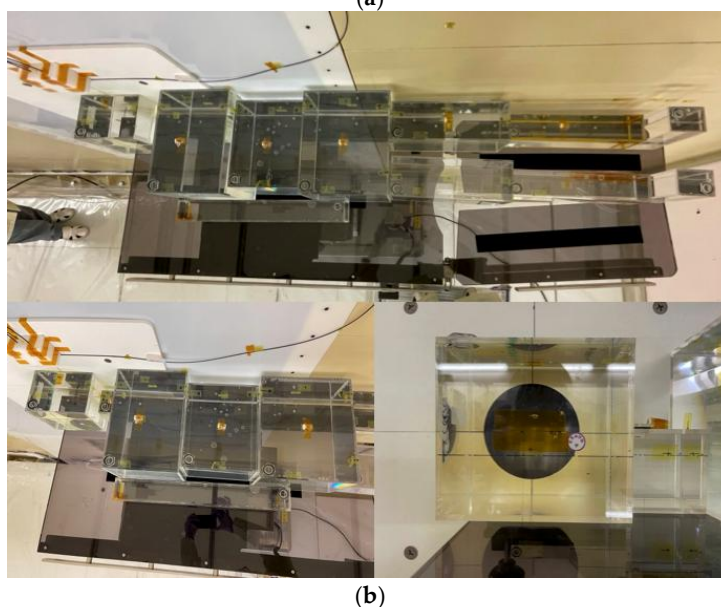
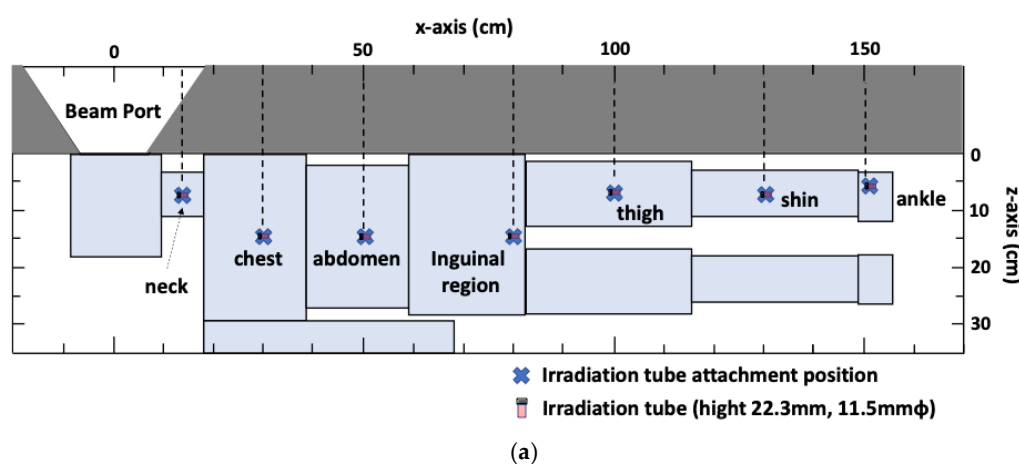


Figure 5. Human phantom configuration for the biological evaluation of out-of-field leakage radiation during BNCT. (a) Schematic diagram showing the attachment positions of the cell-containing

irradiation tubes on the surface of a human whole-body phantom at predefined anatomical regions, including the neck, chest, abdomen, inguinal region, thigh, shin, and ankle. **(b)** Photograph of the experimental setup illustrating the human phantom and fixation of the irradiation tubes using Kapton tape during neutron irradiation.

2.4.6. Quality Assurance and Commissioning of Neutron Irradiation

The quality assurance and commissioning procedures for the radiation leakage dose evaluation were identical to those applied in free-beam experiments. Neutron fluence verification using gold wire activation analysis was performed before and after irradiation to confirm that deviations from the reference values remained within $\pm 5\%$, thereby ensuring the reliability of dose delivery during phantom irradiation experiments.

2.4.7. Reference X-Ray Irradiation

Reference X-ray irradiation was performed to establish a dose–response relationship for micronucleus induction, using the same irradiation system and basic geometry as described in Section 2.3.6, with dose ranges adjusted to the expected leakage dose levels. X-ray irradiation was conducted using the same irradiation geometry as the neutron experiments (Figure 4) at 130 kV and 5 mA with a 0.5 mm aluminum filter. The dose rate was experimentally determined to be 0.47 ± 0.02 Gy/min using thermoluminescent dosimeters with an identical sample geometry. Absorbed doses of 0, 0.2, 0.5, 1, 2, and 4 Gy were delivered, corresponding to irradiation times of approximately 26 s, 1 min 4 s, 2 min 8 s, 4 min 15 s, and 8 min 31 s. These dose points were selected to bracket the expected leakage dose range (approximately 0.3–2 Gy) based on prior studies and to ensure the acquisition of at least four evaluable dose points, including the non-irradiated control.

2.4.8. Micronucleus Formation Assay and Estimation of Leakage Dose

Following irradiation, the cell samples were transported to the biological laboratory and centrifuged to pellet the cells. The supernatant was removed, and the cells were resuspended in fresh culture medium. Cell concentrations were measured in triplicate using an automated cell counter. Cells were seeded into 60 mm dishes containing culture medium supplemented with cytochalasin B (036-17553; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; final concentration: 2.5 $\mu\text{g/mL}$) and incubated for 24–48 h to allow the formation of binucleated cells. After incubation, the cells were harvested, subjected to hypotonic treatment using potassium chloride solution, and fixed with glutaraldehyde solution prepared from 25% glutaraldehyde stock (073-00536; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; concentration: 1%). The fixed cells were stained with Hoechst 33342 (H342; Dojindo Laboratories, Kumamoto, Japan) and mounted on glass slides. Micronuclei were scored in binucleated cells using a fluorescence microscope according to established criteria. For each sample, total micronuclei (T-MN) and induced micronuclei (I-MN) were quantified, with I-MN calculated by subtracting the background values obtained from non-irradiated controls. For neutron-irradiated samples, the I-MN values were converted to estimated leakage radiation doses (GyEq) using the X-ray–derived dose–response relationship.

2.5. Data Analysis and Reproducibility

All clonogenic survival and micronucleus assays were performed as three independent biological replicates ($n = 3$) conducted on separate days, with each replicate derived from an independently prepared cell culture and an independently irradiated sample. For each irradiation condition, the surviving fractions or micronucleus frequencies were calculated for each replicate, and the results are presented as the mean $\pm$ standard error (SE). Survival curves were generated by fitting the experimentally obtained survival data using

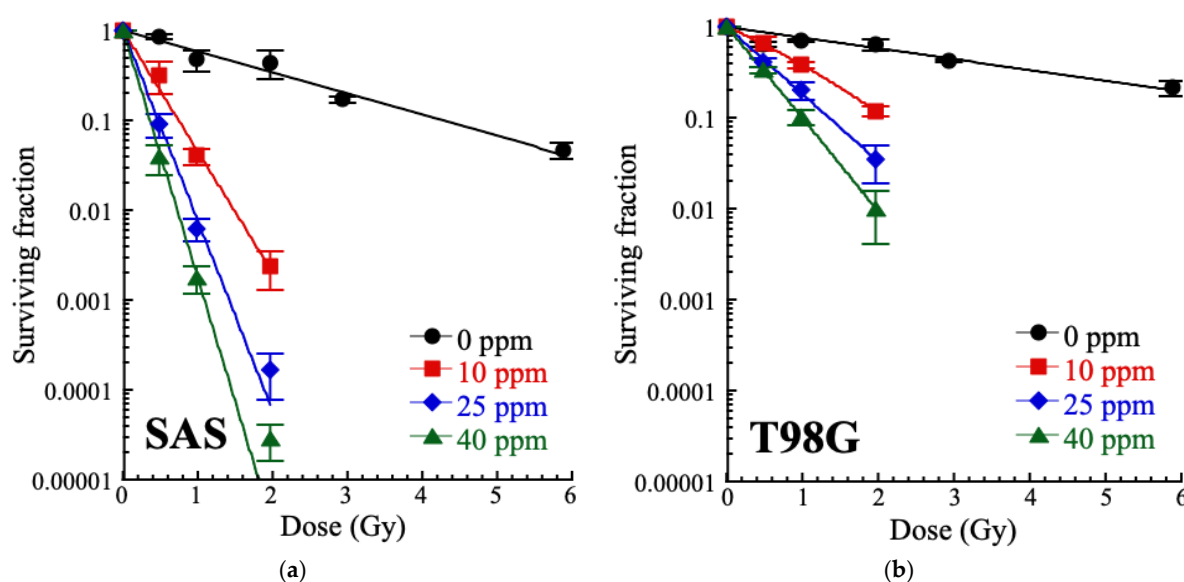
the linear–quadratic (LQ) model for X-ray irradiation and either the LQ or linear model for neutron irradiation, as appropriate. The biological equivalent doses (D_{50} and D_{10}) were derived from the fitted survival curves for each replicate and subsequently averaged. For the micronucleus assays, the induced micronucleus frequencies were calculated by subtracting the background values obtained from non-irradiated controls and converting them to X-ray-equivalent doses using the reference dose–response relationship. The inclusion criteria for data analysis were predefined in the study protocols. Only experiments meeting established quality assurance and biological validity criteria, including stable beam output within $\pm 5\%$, adequate colony formation in control samples, and successful induction of binucleated cells in micronucleus assays, were included in the analysis. No data points were excluded a posteriori. The present study was designed as a preclinical radiobiological characterization of the iBNCT001 system, rather than a hypothesis-testing study. Therefore, no formal statistical hypothesis testing (e.g., p -values or confidence intervals) was conducted. Instead, reproducibility and biological robustness were assessed through independent repeated experiments, controlled irradiation conditions, and consistency of dose–response trends across multiple cell lines and experimental settings, which is a widely accepted approach in BNCT radiobiology and clonogenic survival analyses.

3. Results

3.1. Cell-Based Preclinical Efficacy of BNCT Using iBNCT001

3.1.1. Clonogenic Survival Following BNCT with SPM-011

Clonogenic survival assays were performed to evaluate the cytotoxic efficacy of BNCT using the iBNCT001 system in combination with SPM-011. Survival curves were generated for each cell line by plotting the surviving fraction as a function of the total absorbed dose. Across all tested human cancer cell lines (SAS, T98G, A172, COLO679, and G-361), neutron irradiation in the presence of SPM-011 resulted in a clear dose-dependent reduction in clonogenic survival. The representative survival curves for each cell line are shown in Figure 6. The shapes of the survival curves were consistent with those of high-linear energy transfer radiation, characterized by a steep initial slope and reduced shoulders compared with photon reference irradiation. Notably, inter-cell line differences in radiosensitivity were observed. Among the tested cell lines, SAS cells exhibited the highest sensitivity to BNCT, whereas T98G cells demonstrated relatively lower sensitivity, as reflected by the differences in the survival curve slopes.



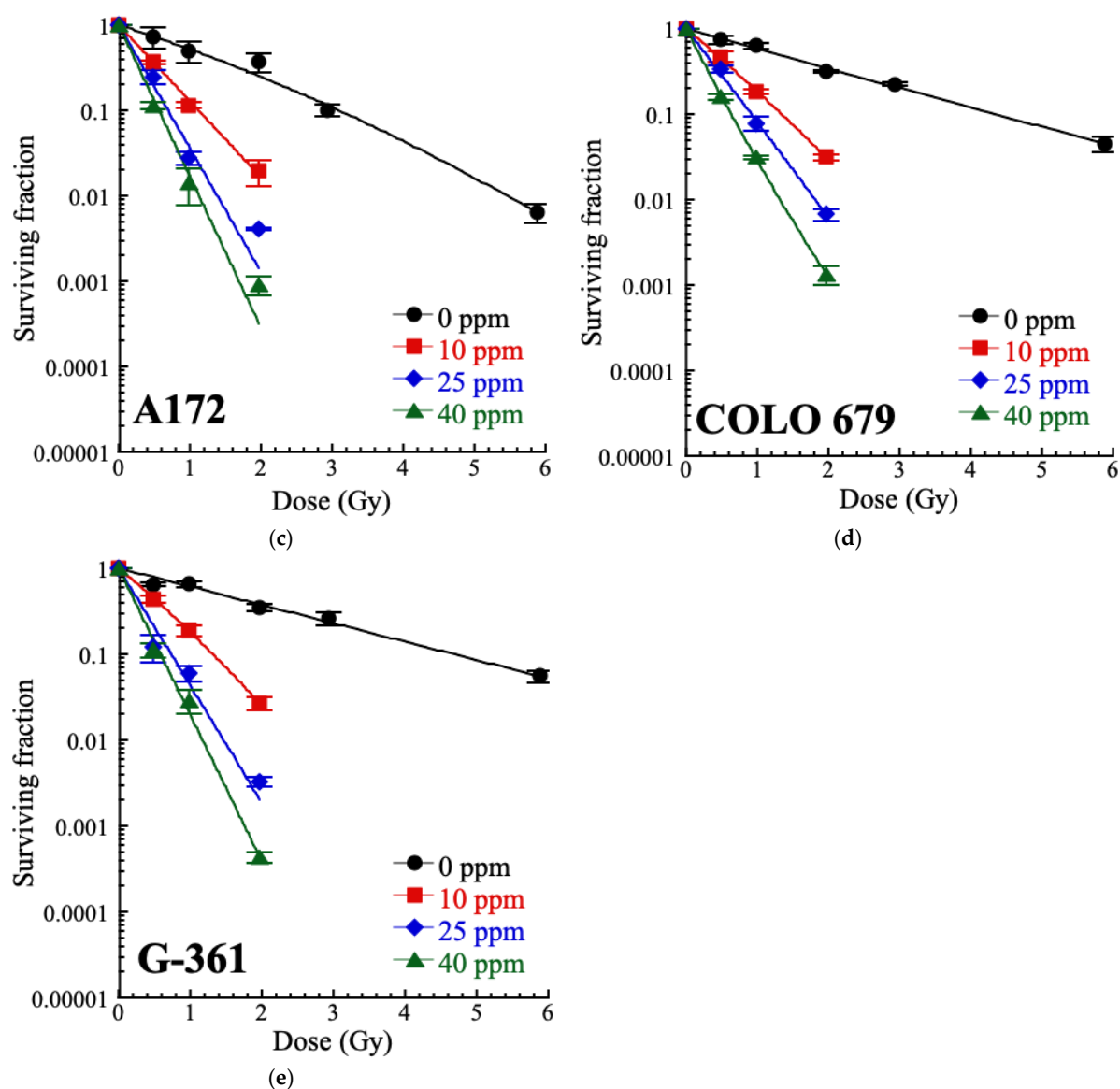


Figure 6. Clonogenic survival curves of (a) SAS, (b) T98G, (c) A172, (d) COLO679, and (e) G-361 cells following BNCT using the iBNCT001 accelerator system in combination with SPM-011. Cells were treated with SPM-011 at ^{10}B concentrations of 0, 10, 25, and 40 ppm before neutron irradiation. The absorbed dose was plotted on a linear scale, and the surviving fraction was plotted on a logarithmic scale. Data points represent the mean of three independent experiments, and the error bars indicate the standard error (SE). Survival curves were fitted using the linear–quadratic (LQ) or linear (L) models, where appropriate.

3.1.2. Determination of Biological Equivalent Dose, D_{50} and D_{10} Values

The absorbed dose required to reduce the surviving fraction to 50% (D_{50}) and 10% (D_{10}) was calculated for each cell line based on fitted survival curves. The resulting D_{50} and D_{10} values are summarized in Tables 1 and 2, respectively. The D_{10} values differed among the cell lines. These results indicate heterogeneity in BNCT sensitivity, which may reflect differences in cellular characteristics, such as boron uptake, DNA repair capacity, and intrinsic radiosensitivity.

Table 1. Biological equivalent dose D_{50} values after BNCT using iBNCT001 neutron combined with SPM-011.

^{10}B Concentration	Biological Equivalent Dose, D_{50} Values (Gy)				
	SAS	T98G	A172	COLO679	G-361
0	1.03 ± 0.14	2.38 ± 0.09	1.39 ± 0.57	1.63 ± 0.44	1.67 ± 0.42
10	0.21 ± 0.01	0.72 ± 0.05	0.44 ± 0.12	0.50 ± 0.10	0.51 ± 0.09
25	0.12 ± 0.01	0.37 ± 0.02	0.17 ± 0.01	0.31 ± 0.04	0.24 ± 0.08
40	0.09 ± 0.01	0.32 ± 0.04	0.13 ± 0.01	0.20 ± 0.02	0.20 ± 0.03

Note: The D_{50} value is shown as the mean and standard error (SE) obtained from three independent experiments. Cells were treated with SPM-011 at ^{10}B concentrations of 0, 10, 25, and 40 ppm before neutron irradiation. Absorbed dose values were calculated based on fitted survival curves using the linear–quadratic (LQ) or linear (L) model, where appropriate. The absorbed dose values included contributions from the boron reaction, nitrogen, hydrogen recoil, and gamma-ray doses. Data are presented as mean $\pm$ standard error (SE) from three independent experiments.

Table 2. Biological equivalent dose D_{10} values after BNCT using iBNCT001 neutron combined with SPM-011.

^{10}B Concentration	Biological Equivalent Dose, D_{10} Values (Gy)				
	SAS	T98G	A172	COLO679	G-361
0	3.84 ± 0.15	8.11 ± 0.38	4.58 ± 1.89	5.66 ± 1.35	5.81 ± 1.29
10	0.71 ± 0.05	2.08 ± 0.08	1.48 ± 0.38	1.62 ± 0.32	1.62 ± 0.32
25	0.41 ± 0.04	1.30 ± 0.15	0.60 ± 0.04	1.03 ± 0.14	0.79 ± 0.26
40	0.32 ± 0.03	0.99 ± 0.08	0.46 ± 0.05	0.69 ± 0.07	0.66 ± 0.10

Note: The D_{10} value is shown as the mean and standard error (SE) obtained from three independent experiments. Cells were treated with SPM-011 at ^{10}B concentrations of 0, 10, 25, and 40 ppm before neutron irradiation. Absorbed dose values were calculated based on fitted survival curves using the linear–quadratic (LQ) or linear (L) model, where appropriate. The absorbed dose values included contributions from the boron reaction, nitrogen, hydrogen recoil, and gamma-ray doses. Data are presented as mean $\pm$ standard error (SE) from three independent experiments.

3.2. Biological Evaluation of Neutron Beam Quality Under Free-Beam Conditions

3.2.1. Clonogenic Survival Curves Following Neutron and X-Ray Irradiation

Clonogenic survival curves were obtained for six cell lines (CHO-K1, SAS, T98G, A172, COLO679, and G-361) following irradiation with fast neutrons generated by the iBNCT001 accelerator system under free-beam conditions and reference X-ray irradiation. For each irradiation dose, the surviving fractions were calculated as the mean of three independent experiments, with the standard error (SE) displayed as error bars. Survival curves were generated by plotting the absorbed dose on a linear scale against the surviving fraction on a logarithmic scale (Figure 7). In all six cell lines, both fast neutron and X-ray irradiation induced a clear dose-dependent reduction in the clonogenic survival.

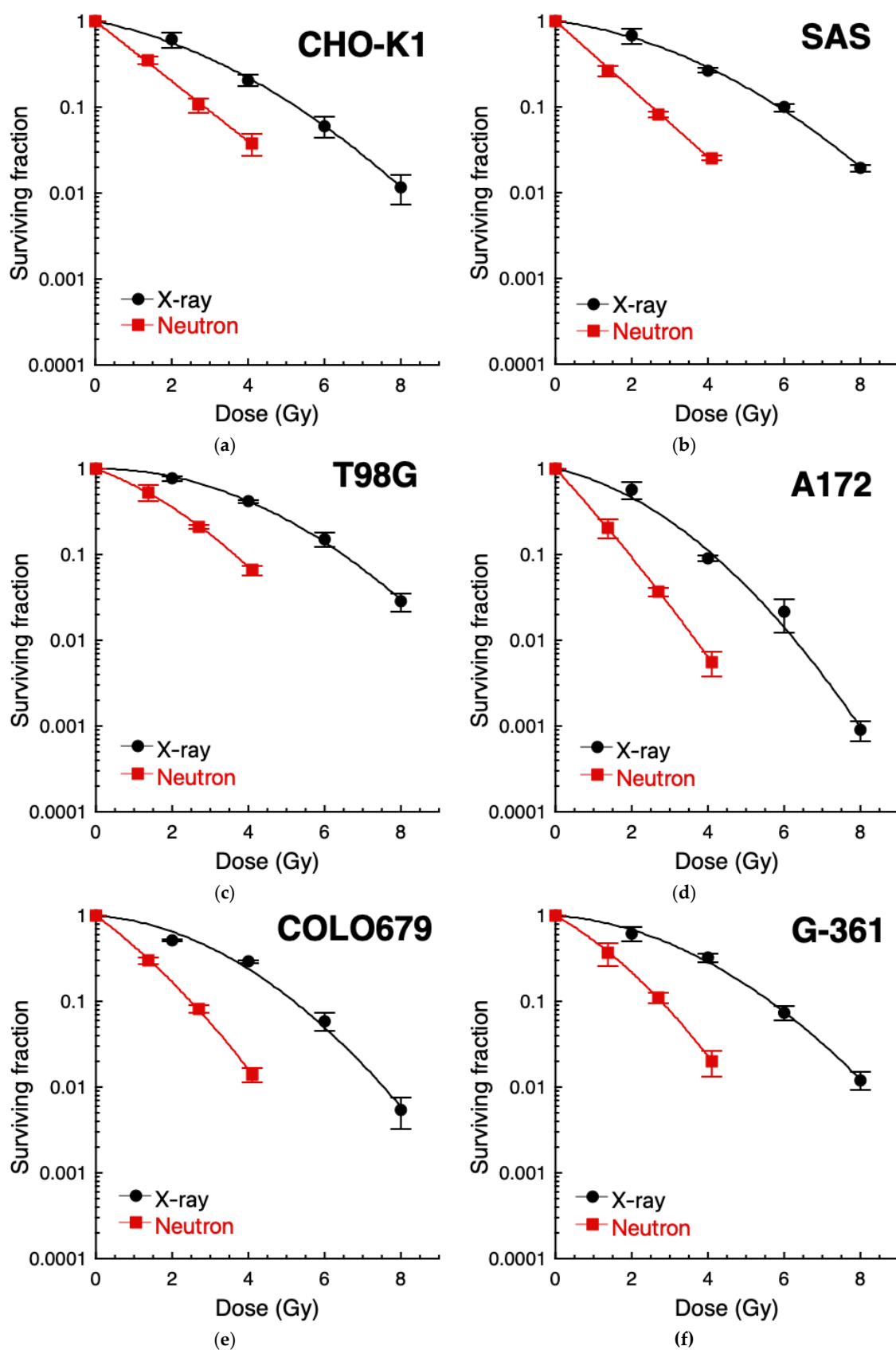


Figure 7. Clonogenic survival curves of (a) CHO-K1, (b) SAS, (c) T98G, (d) A172, (e) COLO679, and (f) G-361 cells following irradiation with fast neutrons generated by the iBNCT001 accelerator system under free-beam conditions and reference X-ray irradiation. The absorbed dose was plotted on a linear scale and the surviving fraction on a logarithmic scale. Data points represent the mean of three independent experiments, with error bars indicating the standard error (SE) of the mean.

Survival curves were fitted using the linear–quadratic (LQ) or linear (L) model, as appropriate, for each radiation quality.

3.2.2. Determination of Biological Equivalent Dose (D_{10}) and Relative Biological Effectiveness (RBE) of Fast Neutrons

The absorbed dose required to reduce the surviving fraction to 10% (D_{10}) was derived from the fitted survival curves for each cell line. Because the neutron beam generated by the iBNCT001 system contained a gamma-ray component, the D_{10} values for fast neutron irradiation were corrected using a gamma contamination ratio of 19.18%, which was determined separately by RPLDs measurements performed under identical irradiation geometry. The corrected D_{10} values for fast neutron irradiation and the corresponding D_{10} values for X-ray irradiation are summarized in Table 3. For all cell lines examined, D_{10} values for fast neutron irradiation were consistently lower than those for X-ray irradiation, confirming the enhanced biological effectiveness of the fast neutron beam.

The RBE was calculated for each cell line using the D_{10} values for X-ray irradiation and gamma-corrected D_{10} values for fast neutron irradiation, thereby minimizing the influence of gamma-ray contamination on the evaluation of neutron biological effectiveness. The calculated RBE values for fast neutrons generated by the iBNCT001 system ranged from 1.96 to 2.72 across the six tested cell lines (Table 3). Among these, the human glioblastoma cell line T98G exhibited the lowest sensitivity to both X-ray and fast neutron irradiation, resulting in the lowest RBE value (1.96). In contrast, the human head and neck squamous cell carcinoma cell line SAS showed relatively high resistance to X-ray irradiation (second most resistant among the six cell lines) and comparatively high sensitivity to fast neutron irradiation (second most sensitive). SAS cells displayed the highest RBE value of 2.72 among the tested cell lines. When averaged across all six cell lines, the mean RBE of fast neutrons generated by the iBNCT001 system was 2.3 (2.29). This value indicates that the biological effectiveness of fast neutron beam irradiation is approximately 2.3-fold higher than that of reference X-ray irradiation.

Table 3. Biological equivalent dose (D_{10}) and relative biological effectiveness (RBE) of fast neutrons generated by iBNCT001 system under free-beam conditions, derived from clonogenic survival analysis with gamma-ray dose correction.

Cells	Biological Equivalent Dose, D_{10} (Gy)		RBE
	X-Ray	Fast Neutron (γ -Corrected)	
CHO-K1	4.60 $\pm$ 0.19	2.28 $\pm$ 0.17	2.02
SAS	5.31 $\pm$ 0.12	1.95 $\pm$ 0.09	2.72
T98G	5.75 $\pm$ 0.20	2.93 $\pm$ 0.05	1.96
A172	3.69 $\pm$ 0.15	1.52 $\pm$ 0.15	2.43
COLO679	4.59 $\pm$ 0.05	2.00 $\pm$ 0.08	2.30
G-361	5.01 $\pm$ 0.24	2.15 $\pm$ 0.20	2.33

Note: D_{10} represents the absorbed dose required to reduce the survival fraction to 10%, derived from the fitted clonogenic survival curves. Because the neutron beam generated by the iBNCT001 system contains a photon component, the fast-neutron D_{10} values were corrected to exclude the gamma-ray contribution. The gamma-ray dose fraction (19.18%) was experimentally quantified under an identical free-beam irradiation geometry using radiophotoluminescence glass dosimeters (RPLDs). Neutron-only D_{10} values were calculated by subtracting the photon contribution from previously reported methodologies. The RBE was calculated as the ratio of the X-ray D_{10} to the gamma-corrected fast-neutron D_{10} . Data are presented as mean $\pm$ standard error (SE) from three independent biological replicates.

3.3. Evaluation of Out-of-Field Leakage Radiation Dose Using a Human Phantom Model

3.3.1. Dose–Response Relationship Between X-Ray Dose and Micronucleus Formation

To establish a reference dose–response relationship for cytogenetic damage, CHO-K1 cells were irradiated with X-rays at doses ranging from 0 to 4.0 Gy, and the micronucleus formation in binucleated cells was quantified. The total number of micronuclei per 1000 binucleated cells (T-MN) and the number of induced micronuclei (I-MN) were evaluated as described in Section 2.4.7 of this paper. At baseline (0 Gy), a mean of 49 micronuclei per 1000 binucleated cells was observed. With increasing X-ray dose, T-MN increased in a dose-dependent manner, reaching 69.3 to 420.3 across the 0.2–4.0 Gy range (Figure 8 and Table 4). Similarly, I-MN increased monotonically with dose, ranging from 20.3 to 371.3 over the same dose range (Figure 8 and Table 4). The relationship between the X-ray dose and micronucleus induction was assessed using curve fitting. While a linear regression model showed a strong fit ($R = 0.99794$), the quadratic model provided the best approximation ($R = 0.99989$) of the dose–I-MN relationship. Therefore, the quadratic fit derived from I-MN was used as the calibration curve for estimating the leakage-equivalent dose (GyEq) from micronucleus induction in the neutron irradiation experiments.

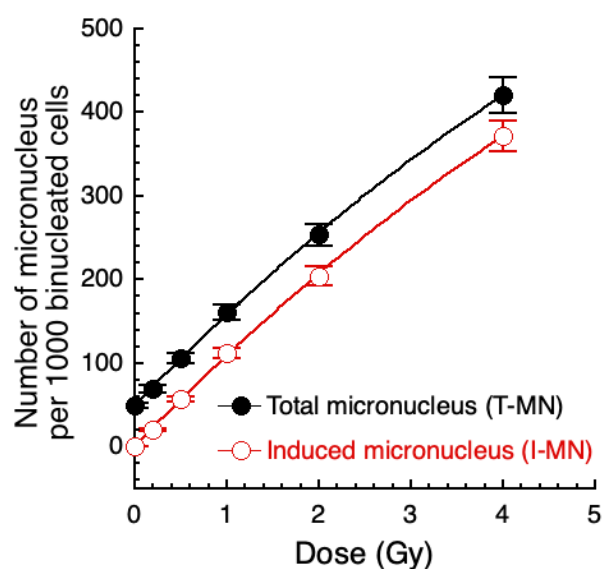


Figure 8. Dose–response relationship between X-ray dose and micronucleus formation in CHO-K1 cells. CHO-K1 cells were irradiated with X-rays at doses ranging from 0 to 4.0 Gy, and micronucleus formation was evaluated using the cytokinesis-blocked micronucleus assay. The number of total micronuclei (T-MN) and induced micronuclei (I-MN) per 1000 binucleated cells is plotted as a function of the X-ray dose. T-MN represents the total number of micronuclei observed, whereas I-MN was calculated by subtracting the background micronucleus frequency in unirradiated controls. Data are presented as mean $\pm$ standard error (SE) from three independent experiments. The dose–I-MN relationship was best fitted using a quadratic regression model and was used for the subsequent estimation of the leakage-equivalent dose in neutron irradiation experiments.

Table 4. Total and induced micronucleus frequencies in CHO-K1 cells following X-ray irradiation.

X-Rays Dose (Gy)	Total Micronucleus (T-MN)	Induced Micronucleus (I-MN)
0	49.0 $\pm$ 2.9	0.0 $\pm$ 0.0
0.2	69.3 $\pm$ 4.4	20.3 $\pm$ 1.7
0.5	106.0 $\pm$ 6.1	57.0 $\pm$ 3.5
1.0	160.7 $\pm$ 9.3	111.7 $\pm$ 6.5
2.0	253.3 $\pm$ 12.6	204.3 $\pm$ 11.0
4.0	420.3 $\pm$ 21.1	371.3 $\pm$ 18.4

Note: Data represent the number of micronuclei per 1000 binucleated cells. Total micronuclei (T-MN) were directly counted, and induced micronuclei (I-MN) were calculated by subtracting the background micronucleus frequency observed in the unirradiated control cells. Values are expressed as mean $\pm$ standard error (SE) from three independent experiments. These data were used to establish an X-ray dose–response calibration curve for estimating the leakage-equivalent dose in neutron irradiation experiments.

3.3.2. Micronucleus Formation in CHO-K1 Cells Exposed to Neutron Irradiation at Different Phantom Locations

To evaluate out-of-field leakage radiation during head irradiation, CHO-K1 cells exposed to SPM-011 (^{10}B = 25 ppm) for 1 h were attached to seven anatomical locations on a human whole-body phantom (neck, chest, abdomen, inguinal region, thigh, shin, and ankle) and irradiated with neutrons generated by the iBNCT001 system under a head-irradiation geometry corresponding to a prescribed tumor dose of 10 GyEq (proton charge 3625 mC, 2.00 mA equivalent; irradiation time of ~31 min). After irradiation, micronucleus formation was quantified, and the leakage-equivalent dose was estimated using the X-ray calibration curve described above. Table 5 summarizes the number of binucleated cells evaluated, observed T-MN, derived I-MN, and estimated leakage-equivalent dose (GyEq) at each phantom location. Among the evaluated locations, the neck, which was closest to the irradiation field, exhibited the highest cytogenetic damage, with a mean T-MN of 184.3 per 1000 binucleated cells. Consistently, the estimated leakage-equivalent dose at the neck was the highest, at 1.31 GyEq (Table 5). In contrast, the remaining locations showed substantially lower micronucleus counts and lower estimated doses. Across the chest, abdomen, inguinal region, thigh, shin, and ankle, the mean T-MN values ranged from approximately 20.0 to 60.0 per 1000 binucleated cells, corresponding to estimated leakage-equivalent doses of approximately 0.18 to 0.54 GyEq (Table 5). These findings indicate a clear spatial gradient in leakage exposure, with the highest estimated dose near the irradiation field and lower estimated doses in more distal body regions.

Table 5. Micronucleus formation and estimated leakage-equivalent dose at different anatomical locations on a human phantom during iBNCT001 irradiation.

Condition or Anatomical Location	Total Micronuclei (T-MN)	Induced Micronuclei (I-MN)	Estimated Leakage-Equivalent Dose (GyEq)
non-irradiation	44.7 $\pm$ 3.5	0.3 $\pm$ 2.8	NA
non-irradiation (with SPM-011)	44.3 $\pm$ 3.8	0.0 $\pm$ 0.0	0.00
neck	184.3 $\pm$ 6.5	140.0 $\pm$ 10.3	1.31
chest	104.3 $\pm$ 7.2	60.0 $\pm$ 4.0	0.54
abdomen	88.0 $\pm$ 5.5	43.7 $\pm$ 7.2	0.39
inguinal region	81.7 $\pm$ 5.0	37.3 $\pm$ 3.2	0.33
thigh	86.0 $\pm$ 4.4	41.0 $\pm$ 2.9	0.37
shin	73.3 $\pm$ 5.0	31.0 $\pm$ 1.5	0.28
ankle	65.0 $\pm$ 3.8	20.0 $\pm$ 1.2	0.18

Note: CHO-K1 cells exposed to SPM-011 (^{10}B concentration of 25 ppm) were attached to different anatomical locations on a human whole-body phantom and irradiated with neutrons generated by the iBNCT001 system under head irradiation geometry corresponding to a prescribed tumor dose of 10 GyEq. Total micronuclei (T-MN) and induced micronuclei (I-MN) were quantified per 1000 binucleated cells using a cytokinesis-block micronucleus assay. The I-MN values were calculated by subtracting the background micronucleus frequency obtained from the unirradiated controls. The leakage-equivalent dose (GyEq) was estimated by converting the I-MN values using the X-ray dose–

response calibration curve shown in Figure 8. Data are presented as mean values from three independent experiments.

4. Discussion

Accelerator-based boron neutron capture therapy (AB-BNCT) has progressed from experimental neutron sources to clinically deployable systems through the development of compact accelerators, optimized neutron production targets, and integrated treatment-planning methodologies. Recent reports from Japan and other regions have emphasized that the successful clinical translation of AB-BNCT requires not only sufficient neutron intensity but also validated radiobiological characteristics, including boron-dependent cytotoxicity, reproducible RBE, and acceptable levels of out-of-field exposure under clinically realistic irradiation geometries [7–9].

In the present study, three preclinical investigations were integrated to establish the efficacy, radiobiological validity, and safety of the linac-based iBNCT001 system in combination with a clinically approved boron agent, SPM-011. Collectively, these experiments were designed to address key translational questions: whether the neutron beam combined with SPM-011 induces boron concentration- and dose-dependent cytotoxicity, whether the fast-neutron component exhibits RBE values consistent with established AB-BNCT systems, and whether out-of-field exposure remains sufficiently low to support safe clinical application. In this section, we first interpret the biological efficacy results obtained with iBNCT001 in comparison with previously reported accelerator-based BNCT systems, then discuss the radiobiological characteristics of the fast-neutron component, and finally address safety considerations and limitations of the present study.

In the cell-based efficacy study, neutron irradiation in the presence of SPM-011 produced clear ^{10}B concentration- and dose-dependent reductions in clonogenic survival across multiple tumor cell lines. This finding confirms that the observed cytotoxicity is primarily driven by the boron neutron capture reaction rather than nonspecific photon contamination. Similar boron-dependent enhancement of cell killing has been reported in previous BNCT preclinical studies using accelerator-based neutron sources and clinically relevant boron concentrations, supporting the biological plausibility of the present results [19,32,33].

Importantly, when compared with previously reported AB-BNCT platforms, the magnitude and trends of the observed cytotoxic effects were comparable to those reported for earlier systems, including the cyclotron-based epithermal neutron source (C-BENS) and compact intense neutron source (CICS-1). These systems have demonstrated reproducible boron-dependent biological effects *in vitro* and *in vivo*, forming the basis for subsequent clinical translation [7,12,17]. From this perspective, the efficacy data obtained with iBNCT001 can be regarded as biologically equivalent and clinically adequate rather than system-specific outliers.

A free-beam experiment was conducted to further characterize the radiobiological properties of the fast-neutron component generated by iBNCT001. The steeper survival curves and reduced shoulder regions observed under fast neutron irradiation compared with reference X-ray exposure suggest a diminished capacity for sublethal damage repair, which is a well-recognized characteristic of high-LET radiation. This tendency was consistently observed across all tested cell lines and was consistent with the higher biological effectiveness of fast neutrons relative to photons. Using six different cell lines and clonogenic survival analysis, the fast-neutron RBE values were consistently approximately 2, with an average representative value of approximately 2.3. These values are in close agreement with previously reported fast neutron RBE values obtained using similar biological endpoints and experimental geometries. In particular, C-BENS has been reported to yield fast-neutron RBE values of approximately 2.4, whereas CICS-1 using a lithium target has

demonstrated RBE values of approximately 2.0 [7,16]. The iBNCT001 results fell between these values, indicating that the neutron beam quality was radiobiologically consistent with that of the established AB-BNCT systems.

Because accelerator-based neutron beams inevitably include a photon component, careful consideration of the photon admixture is essential when evaluating the fast-neutron RBE. In the present study, the photon contribution in free-beam geometry was experimentally quantified, and the neutron D_{10} values were corrected accordingly prior to the RBE calculation. This correction ensures that the reported RBE reflects the biological effectiveness of the fast-neutron component, minimizing bias arising from mixed-field exposure. This approach is consistent with the best practices recommended in recent radiobiological and dosimetric evaluations of AB-BNCT systems [28,29,34]. The present study did not aim to benchmark iBNCT001 against other BNCT systems through direct side-by-side biological experiments, as such comparisons would require identical irradiation geometries, dose rate conditions, and biological protocols across facilities, which is not practically feasible. Instead, the fast-neutron RBE values obtained in this study were evaluated using a standardized D_{10} -based methodology with gamma-ray correction, which has been widely adopted in previous accelerator-based BNCT studies. In particular, similar RBE evaluation frameworks have been reported for both beryllium-target cyclotron-based systems (C-BENS) and lithium-target linac-based systems (CICS-1), enabling indirect but meaningful comparisons in the literature [28,29]. The consistency of the RBE values observed for iBNCT001 with those reported in these established systems supports the validity of the present biological evaluation, rather than indicating isolated or insufficient benchmarking of the experimental conditions. It should be emphasized that the RBE values reported in this study were derived after explicitly correcting for the gamma-ray component contained in the free neutron beam. The gamma-ray fraction (19.18%) was experimentally quantified under an identical irradiation geometry, and the neutron-only D_{10} values were calculated by subtracting the photon contribution, following the methodology described in previous reports [28,29]. This correction is critical because even modest photon contamination can substantially influence the apparent RBE values, particularly under free-beam neutron irradiation conditions. Therefore, the RBE values obtained in this study represent the intrinsic biological effectiveness of the neutron component generated by the iBNCT001 system, enabling a more reliable comparison with previously reported accelerator-based BNCT neutron sources.

Safety considerations were addressed in an out-of-field exposure study that employed a humanoid phantom and a biological dosimetry approach based on micronucleus induction in CHO-K1 cells. By converting the micronucleus frequency to an X-ray-equivalent dose using a calibrated dose–response relationship, this study provided an integrated biological assessment of leakage radiation under a clinically relevant head-irradiation configuration. The estimated out-of-field doses decreased markedly with increasing distance from the irradiation field and remained low at distal anatomical sites.

These findings are consistent with those of previous experimental and computational studies evaluating out-of-field exposure in BNCT and other advanced radiotherapy modalities. Monte Carlo-based analyses and experimental measurements have demonstrated that, when appropriate collimation and shielding are employed, leakage doses during head irradiation are substantially lower than thresholds associated with deterministic tissue reactions [35–38]. From a clinical standpoint, the present biological dosimetry results support the feasibility of safely delivering BNCT using iBNCT001 without inducing clinically significant out-of-field toxicities.

Taken together, the three non-clinical studies demonstrate that iBNCT001 combined with SPM-011 satisfies the key translational requirements for AB-BNCT: boron-dependent cytotoxic efficacy, fast-neutron RBE values consistent with established systems, and low

out-of-field biological dose under clinically relevant conditions. While further *in vivo* and clinical investigations are required to fully define the therapeutic benefits and long-term safety, the present results provide a robust preclinical foundation supporting the clinical feasibility and relevance of iBNCT001-based BNCT.

Several limitations of the present study should be acknowledged when interpreting the results. First, the present study is based on *in vitro* cell-based experiments, which provide a controlled and quantitative framework for radiobiological evaluation but cannot fully reproduce the complexity of boron pharmacokinetics, tissue heterogeneity, and microenvironmental effects observed *in vivo*. Comprehensive *in vivo* non-clinical studies addressing both efficacy and safety have already been completed under GLP-compliant conditions and submitted to the Pharmaceuticals and Medical Devices Agency (PMDA) as part of the regulatory review process; however, these results have not yet been published and will be reported separately in the future. Second, boron concentration-dependent cytotoxicity was evaluated using the nominal boron concentration in the culture medium, rather than direct intracellular boron quantification. This approach is commonly employed in BNCT radiobiology studies when the primary objective is to assess neutron beam characteristics and relative biological effectiveness under standardized conditions rather than boron pharmacokinetics [39,40]. Similar experimental designs using culture medium boron concentrations have been adopted in previous *in vitro* BNCT studies to evaluate boron-dependent cell killing and enhancement effects [33,41,42]. However, because all experiments were conducted under identical exposure conditions with respect to boron concentration, incubation time, and irradiation geometry, the observed dose-response trends and comparative analyses primarily reflect differences in neutron beam characteristics and intrinsic cellular radiosensitivity rather than variability in boron delivery. Accordingly, although absolute comparisons of boron uptake between cell lines were beyond the scope of this study, the use of nominal medium concentrations was sufficient for the present purpose of system-oriented radiobiological validation. Despite these limitations, the present study provides an independent and systematic *in vitro* biological validation of the iBNCT001 accelerator system, serving as an essential preclinical step prior to and complementary to ongoing *in vivo* and clinical investigations.

5. Conclusions

This study provides an integrated *in vitro* preclinical biological evaluation of the linac-based iBNCT001 system combined with the clinically approved boron compound SPM-011. The results demonstrated consistent boron concentration- and dose-dependent cytotoxicity, clinically relevant fast neutron RBE characteristics, and low out-of-field biological doses. While the present findings support the biological feasibility and safety profile of iBNCT001-based BNCT, further *in vivo* efficacy, biodistribution, and treatment planning-oriented evaluations are required to fully establish its clinical utility.

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Abbreviations

The following abbreviations are used in this manuscript:

BNCT	Boron Neutron Capture Therapy
AB-BNCT	Accelerator-Based Boron Neutron Capture Therapy
RBE	Relative Biological Effectiveness
Gy	Gray
GyEq	Gray Equivalent
^{10}B	Boron-10
E-MEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
PBS	Phosphate-Buffered Saline
LQ model	Linear–Quadratic model
T-MN	Total Micronucleus
I-MN	Induced Micronucleus
C-BENS	Cyclotron-Based Epithermal Neutron Source
CICS-1	Cancer Intelligence Care Systems-1

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