

BNCT Pancreatic Cancer Treatment Strategy with glucose-conjugated boron drug

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Abstract

Multidisciplinary therapy centered on radical surgery for resectable pancreatic cancer is expected to prolong prognosis, but relies on CA19-9 biomarker levels to determine treatment strategy. Boron neutron capture therapy (BNCT) is a chemoradiotherapy using tumor hyperaccumulator boron drugs and neutron irradiation. The purpose of this study is to investigate novel boron drug agents for BNCT for pancreatic cancer.

Bioinformatics was used to evaluate the uptake of current boron amino acid (BPA) drugs for BNCT into pancreatic cancer. The expression of the amino acid transporter LAT1, a BPA uptake transporter, was low in pancreatic cancer and even lower in high CA19-9 pancreatic cancer. In contrast, the glucose transporter was high in high CA19-9

pancreatic cancers and inversely correlated with LAT1 expression. Considering the low EPR effect in pancreatic cancer, we synthesized a small molecule Glucose-BSH, which is boron BSH bound to glucose, and confirmed its specific uptake in pancreatic cancer. uptake of Glucose-BSH was confirmed in an environment compatible with the tumor microenvironment. The therapeutic efficacy and safety of Glucose-BSH by therapeutic neutron irradiation were confirmed with BNCT. We report Glucose-BSH boron drug discovery study of a Precision Medicine BNCT with application to high CA19-9 pancreatic cancer.

INTRODUCTION

Boron neutron capture therapy (BNCT) was proposed by Dr. Gordon Locher in 1936 as a treatment that induces cell-killing effects by the nuclear reaction of boron atoms (^{10}B) and neutrons[1, 2]. In Japan, basic and clinical BNCT research was initiated at the Kyoto University Research Reactor Institute (KURRI) [3, 4]. Accelerator-based neutron sources that could be installed in hospitals and the development of boron drugs were pursued at facilities around the world[5]. Because the range of He particles and Li nuclei produced by the boron neutron capture reaction is within 10 μm , it is important to specifically introduce boron ^{10}B -containing agents inside target cancer cells[6].

The drugs currently used for BNCT include p-boronophenylalanine (BPA) and sodium borocaptate (BSH) [7]. BPA, which has been approved by the pharmaceutical industry, is an amino acid derivative boron drug; BPA is a boron atom (^{10}B) bonded to phenylalanine. Phenylalanine is an amino acid that is a substrate for melanin synthesis and was created as a drug targeting melanoma, which is an active melanin synthesizing disease[8, 9]. BPA uptake has been confirmed in many cancer cells and is mediated by an amino acid transporter (LAT1) that is highly expressed in cancers[10-12]. In 1987, the first clinical success of BNCT for malignant melanoma was achieved by Mishima et al., leading to the world's first insurance approval in Japan for recurrent head and neck cancer in 2020[13, 14].

Pancreatic cancer has one of the poorest prognoses among malignant tumors[15, 16]. Even at stage I, pancreatic cancer has a 5-year survival rate of only 40%–50%[17, 18]. Only surgery for early resectable stage IA pancreatic cancer has a good 5-year survival rate, of 80% or higher, and thus a treatment strategy that includes early detection and radical surgery is important[19]. CA 19-9 is a tetrasaccharide based on galactose, N-acetylglucosamine, sialic acid, and fucose[20], and was first discovered in 1979 through research using a monoclonal antibody to isolate tumor-associated antigen in colon cancer, and was found to be produced in pancreatic cancer in 1981[21, 22]. Since the development of a radioimmunoassay to measure CA19-9 levels in 1983[23], CA19-9 has rapidly developed as a biomarker and is currently highly sensitive (70%-90%) as a diagnostic marker for pancreatic cancer, and many reports have shown a correlation with prognosis[15].

Reports on the relationship between blood CA19-9 levels and prognosis in patients with localized pancreatic cancer who underwent resection have described poor prognoses dependent on CA19-9 levels[24-27]. Current guidelines recommend resection after preoperative chemotherapy for localized pancreatic cancer[28]. Studies have reported that patients with localized pancreatic cancer with elevated levels of CA19-9 are at high risk of postoperative recurrence because of micrometastases and these patients are

advised to avoid surgery[29]. The mechanism linking high CA19-9 levels and poor prognosis has remained unclear. One possibility is that CA19-9 forms a complex with the extracellular matrix and that this complex is responsible for the increased risk of recurrence. One study reported that CA19-9 forms a complex with extracellular matrix and is involved in carcinogenesis through activation of the RAS pathway via EGFR[30, 31]. CA19-9 levels in localized pancreatic cancer are a decisive factor in determining whether radical surgery can be performed, making research on high CA19-9 level pancreatic cancer important. Thus, there is a need to develop therapies effective for high CA19-9 expressing cancers.

Research on a variety of monosaccharide-binding agents has been underway since the early 1990s, and an agent that binds to antiretroviral agents to glucose was reported in 1991[32]. Since then, reports showing that the glucose transporter (GLUT-1) is overexpressed in the majority of cancers of various tissue origin have led to research on glucose-binding agents, including glufosfamide, a pioneer in sugar-binding anticancer drugs[33]. Further research in this area is expected to develop in 2020 and beyond, with reports of glucose-binding agents to be applied to BNCT[33-36]. In this study, we synthesized a novel boron agent,

1,2,3,4,5,6,7,8,9,10,11-undecahydro-12-(1-thio- β -Dglucopyranose)-dodecaborate

(Glucose-BSH), by binding glucose to boron BSH. We investigated the potential utility of Glucose-BSH as preoperative BNCT therapy for pancreatic cancer with high CA19-9.

Methods

Ethics Statement

All animal use and care procedures were conducted in accordance with ARRIVE 2.0 guidelines, approved by the Department of Animal Resources, Advanced Science Research Center, Okayama University (OKU-2020533, 2020706, 2021548) and the Kyoto University Institute for Integrated Radiation and Nuclear Science (KURNS) ethics committee (KURNS approval number 2021-38, 2022-38) and followed all required protocols.

Mice used in the experiment were reared in plastic cages from Clare Japan Inc. using a positive pressure unidirectional airflow rearing rack (AP Anicon by Seiken Corporation) under the following conditions: temperature 20°C to 25°C, humidity 40% to 60%, ventilation in the cage at least 50 times/hour, light/dark cycle 12/12 hours, and lighting at 8:00 AM. The maximum caging density was within standards according to cage size. As bedding, animal bedding paper shavings (Eco Chip CL-4163, CLEA Japan, Inc) were provided. Mice were fed a standardized mouse diet (MF, Oriental Yeast Co., LTD) and provided drinking sterile water ad libitum. All materials, including IVCs, lids, feeders, bottles, bedding, and water were autoclaved before use. All mice were negative for at least all relevant murine infectious agents.

The protocol for pancreatic cancer imaging evaluation was approved by the Okayama University and Okayama University Hospital Ethics Committee (approval number: OU-research 2004-028). Patient data were used for simulation with personal data erased.

Synthesis of the boron agents

All experiments were performed under an atmosphere of nitrogen. Solvents were dried by standard methods and distilled prior to use. High-performance liquid chromatography (HPLC) was performed on a Japan Analytical Co. model LC-918V

using a JAIGEL GS-320 (eluent: methanol). CD₃OD and D₂O were purchased from Cambridge Isotopes. NMR spectra were recorded on a Varian NMR System 400-MR ASW and a Varian NMR System PS600. The ¹H NMR shifts are given in ppm relative to tetramethylsilane (TMS; δ = 0). IR spectra were obtained on a Thermo Nicolet IR Affinity-1 spectrophotometer. Electrospray ionization mass spectrometry (ESI-MS) spectra data were obtained on a Bruker Daltonics micrOTOF II spectrometer. Reagents were purchased from Wako Pure Chemical Industries Ltd., Tokyo Chemical Industries Co., Ltd., Kanto Kagaku Co., Ltd., Nacalai Tesque Co., Ltd., and Aldrich Chemical Co.

Synthesis of 1,2,3,4,5,6,7,8,9,10,11-Undecahydro-12-(1-Thio-β-D-Glucopyranose 2,3,4,6-Tetraacetato-S)-Dodecaborate (Acetylated Glucose-BSH)

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (300 mg, 0.73 mmol) was dissolved in acetonitrile (30 mL) and triethylamine (2.5 mL, 22 mol) and Na₂¹⁰B₁₂H₁₁SH (230 mg, 1.09 mmol) was added. The reaction mixture was stirred for 24 h at 80°C under a nitrogen atmosphere. After removal of the solvent under reduced pressure, the residue was solved in H₂O and washed with CHCl₃. After removal of the solvent under reduced pressure, Acetylated Glucose-BSH (0.105 mg, 0.15 mmol) was obtained as a transparent oil in 20% yield. In the case of ¹H and ¹³C NMR spectra, datum of [(HNEt₃)₂]²⁺[Glucose-BSH Acetyl protection]²⁻ was shown. Because ¹³C NMR spectrum of 2Na⁺[Glucose-BSH Acetyl protection]²⁻ showed the low signal-to-noise ratios spectra probably due to formation of micelles in H₂O. Acetylated Glucose-BSH: ¹H NMR (600 MHz, D₂O) δ 0.78-1.55 (m, 11H), 1.147 (t, J = 7.2 Hz, 18H), 1.91 (s, 3H), 1.95 (s, 3H), 1.98 (s, 3H), 2.00 (s, 3H), 3.07 (q, J = 7.2 Hz, 18H), 3.81 (ddd, J = 10.2 Hz, 5.4 Hz, 2.0 Hz, 1H), 4.04 (dd, J = 12.0 Hz, 2.0 Hz, 1H), 4.20 (dd, J = 12.6 Hz, 5.4 Hz, 1H), 4.34 (dd, J = 12.3 Hz, 5.0 Hz, 1H), 4.70-4.73 (m, 2H), 4.88 (t, J = 10.2 Hz, 1H), 5.13-5.18 (m, 1H); ¹³C NMR (125 MHz, D₂O) δ 8.3, 20.1, 20.4, 20.6, 22.0, 46.7, 62.5, 68.9, 72.8, 74.7, 85.9, 172.9, 173.0, 173.2, 174.0; IR (KBr): 2981, 2932, 2678, 2493, 1731, 1380, 1229, 1036 cm⁻¹. MS (ESI). *m/z* calcd for C₁₄H₃₀¹⁰B₁₂Na₃O₉S 563.2856 [M+Na]⁺, found 563.2917 [M+Na]⁺.

Synthesis of 1,2,3,4,5,6,7,8,9,10,11-Undecahydro-12-(1-Thio-β-D-Glucopyranose)-Dodecaborate (G-BSH)

Sodium methoxide (30 mg, 0.56 mmol) was added to a methanol solution (12 mL) of Acetylated Glucose-BSH (70 mg, 0.14 mmol) at r.t.. The reaction mixture was stirred at the same temperature for 6 h. DOWEX™ 50W×8 100-200 mesh (H) cation

exchange resin was added and the solution was stirred until neutral. The mixture was filtered, and then concentrated to give Glucose-BSH (40 mg, 0.11 mmol) was obtained as a transparent oil in 80% yield. Glucose-BSH: ^1H NMR (400 MHz, CD_3OD) δ 0.45-2.20 (m, 11H), 3.07 (t, $J = 9.2$ Hz, 1H), 3.20-3.41 (m, 3H), 3.57 (dd, $J = 11.6$ Hz, 9.6 Hz, 1H), 3.86 (dd, $J = 12$ Hz, 2.4 Hz, 1H); ^{13}C NMR (125 MHz, D_2O) δ 61.3, 70.0, 74.5, 77.3, 79.3, 87.6; MS (EMI-MS). m/z calcd for $\text{C}_6\text{H}_{22}^{10}\text{B}_{12}\text{Na}_3\text{O}_5\text{S}$ 395.2433 $[\text{M}+\text{Na}]^+$, found 395.2440 $[\text{M}+\text{Na}]^+$.

Cell lines and cell culture

Human pancreatic cancer cell lines BxPC-3, S2-028, PANC-1 were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University (Japan). Panc0504, Panc0403, QGP1 were obtained from ATCC (Manassas, VA). BxPC-3, S2-028, QGP1 were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum 1% penicillin-streptomycin. Panc0504, Panc0403 were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum 1% penicillin-streptomycin and 0.1% human insulin solution (Sigma, I9278). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C .

CA19-9 enzyme-linked immunosorbent assay

CA19-9 secretion was measured using the CA19-9 ELISA Kit (EIA5069R, Cosmo Bio, Tokyo, Japan) according to the manufacturer's instructions.

Plates were coated with PAA-conjugated glycans or 19-9 glycoprotein, $10\text{ }\mu\text{g/mL}$ in 0.05 M Na-carbonate buffer, pH 9.6, for 1 h at 37°C , followed by blocking with 3% BSA in PBS (0.01 M Na_2HPO_4 , 0.01 M NaH_2PO_4 , 0.138 M NaCl , 0.0027 M KCl , pH 7.4) for 1 h at 37°C . Plates were washed three times with PBS containing 0.1% Tween-20 (PBS-0.1%). mAbs 58/1 and 58/2 (in 2-fold dilution starting from $1:10^3$; $100\text{ }\mu\text{L}$ per well) were added and the plates were incubated for 1 h at 37°C and washed three times with PBS-0.1%. Anti-mouse Ig-HRP conjugate (1:2000 dilution in PBS containing 0.3% BSA) were added and the plates were incubated for 1 h at 37°C . After washing with PBS-0.1%, the color was developed by 30-min incubation with 0.1 M sodium phosphate/ 0.1 M citric acid buffer, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2 , and the reaction was stopped with the addition of $50\text{ }\mu\text{L}$ 1 M H_2SO_4 . Absorbance was recorded at 492 nm using a microtiter plate reader (Multiskan MCC/340; Labsystems, Finland). The results were presented as mean value with standard deviation.

Immunocytochemistry (ICC) of human pancreatic cells and Orthotopic mouse model of pancreatic cancer

Immunocytochemistry (ICC) was observed with by confocal laser microscope (FV1200, Olympus, Japan) with using a previously described method [\[\[37\]\]](#).

BxPC-3, panc0504, S2-028, and panc0403 were used as CA19-9 producing pancreatic cancer cells, and PANC-1 and QGP-1 as CA19-9 nonproducing pancreatic cancer cells. After 24 hours of cell culture, the cells were thoroughly washed five times with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min, and incubated with the primary antibody mouse anti-CA19-9 mAb (1st antibody, 1/100 dilution, ab3982, abcam). The secondary antibody was incubated with mouse IgG conjugated Alexa-Fluor 568 (2nd antibody, ab175473, 1/100 dilution, abcam) for 2 h. Nuclear staining with Hoechst 36983 was carried out for 30 min.

The primary antibody, mouse BSH antibody, was kindly provided by Prof M. Kirihata, Osaka Metropolitan University. BxPC-3, panc0504, and panc0403 were used as CA19-9 producing pancreatic cancer cells and PANC-1 as CA19-9 non-producing pancreatic cancer cells. In brief, after incubation with Glucose-BSH 100 μ M, the cells were thoroughly washed five times with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min, and incubated with the primary antibody mouse anti-BSH mAb(1st antibody, 1/1000 dilution, given by Prof. Kirihata in Osaka Metropolitan University). The secondary antibody was incubated with mouse IgG conjugated Alexa-Fluor 568 (2nd antibody, ab175473, 1/100 dilution, abcam) for 2 h. Nuclear staining with Hoechst 33342 and cytoplasmic staining with Phalloidin 488 were carried out for 30 min. Fresh-frozen 5 μ m sections of tumors were used for immunohistochemistry of samples collected from mice. Tumor sections were stained with anti-HLCA A antibody (ab52922) as primary antibody, Alexa-Fluor 488 (ab150077) as secondary antibody, and Nuclear Blue™ DCS1 for nuclear staining.

Western blot analysis

Proteins extracted from whole-cell lysates were separated by electrophoresis on 10%–15% SDS-polyacrylamide gels and transferred onto Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare UK, UK). The membranes were incubated with primary Abs to GLUT1 (ab15309, Abcam, Cambridge, MA, USA), GLUT3 (ab15311, Abcam, Cambridge, MA, USA), LAT1 (ab208776, Abcam,

Cambridge, MA, USA), LAT2 (ab233088, Abcam, Cambridge, MA, USA), and β -Actin (Sigma-Aldrich, Saint Louis, MO, USA), followed by peroxidase-linked secondary Ab. The Amersham ECL chemiluminescence system (GE Healthcare UK) was used to detect the peroxidase activity of the bound Ab. Equal loading of samples was confirmed using β -Actin.

Inductively coupled plasma (ICP) measurement of intra-cellular boron uptake

All of the cell samples were measured by ICP-MS (Agilent 7900 ICP-MS, Agilent Technologies Inc., Santa Clara, CA) using a previously described method [9]. The intracellular boron concentration (^{10}B ng/ 10^6 cells) was calculated from the amount of boron present and the cell number of the sample. The ICP was measured at Okayama University Institute of Plant Science and Resources.

Evaluation of cell viability under different glucose concentrations in culture and with GLUT inhibitors

To culture at different glucose concentrations, medium with glucose concentrations of 2 g/L (RPMI-1640, Gibco) was diluted with PBS to create 0.25, 0.5, 0.75, 1.0, and 2 g/L medium, and cell viability was evaluated at 24 and 48 hours after culture medium replacement by absorbance (450 nm) measurement.

For evaluation of intracellular boron uptake, 0.5, 2, and 4.5 (08458-45, Nacalai, Japan) g/L medium was used, and cells were collected 48 hours after medium replacement and boron concentration was measured by ICP.

WZB117 (AG-CR1-3694, KOM) was used as the GLUT inhibitor, and the inhibitor was dosed into the medium at 24 hours after the start of culture at a glucose concentration of 2.0 g/L. Samples 24 hours after dosing WZB117 were collected and boron concentrations were measured by ICP.

Mouse pancreatic tumor model with pharmacokinetics (PK)

All animal use and care procedures were approved by the Department of Animal Resources, Advanced Science Research Center, Okayama University (OKU-2020706, OKU-2021456, OKU-2021515) and followed all required protocols. Athymic mice were used (BALB/C-nu/nu, female, 6–8 weeks, 16–20 g, Japan CLEA, Inc.). BALB/c nude mice were inoculated subcutaneously with panc0403 cells under 2% isoflurane inhalation into the right lateral abdomen to create panc0403 tumor models (4×10^6 cells per mouse). One week after transplantation, the tumor formation was confirmed to be 20–60 mm³ (no significant difference in each group), Glucose-BSH (75mg/kg) diluted

by ultrapure water. Was intraperitoneally administered into the mouse under 2% isoflurane inhalation. The mice were euthanized 0.5, 1, 2, 3, 6 or 12 hours after administration, and tumors and organs were collected. We also created a panc0403 orthotopic tumor model by transplanting panc0403 cells under the pancreatic capsule by opening mice under 2% isoflurane inhalation (1×10^6 cells per animal). Three weeks after transplantation, a 320 mm³ tumor was found to have formed under the anterior capsule of the pancreas. Glucose-BSH (75 mg/kg) diluted by ultrapure water. Was administered intraperitoneally under 2% isoflurane inhalation. Mice were euthanized 0.5, 1, 2, or 4 hours after administration, and tumors and organs were collected. The collected samples were soaked in 1 ml of 70% nitric acid and ashed at 90°C. The ashed samples were diluted with water, and the volume was adjusted to 10 ml. After filtering the samples with a syringe filter (0.5 µm), the amount of boron in the samples was quantified using ICP-MS (Agilent 7900 ICP-MS, USA).

Neutron radiation experiment at the Institute for Integrated Radiation and Nuclear Science, Kyoto University (KURNS)

All protocols were approved by the KURNS ethics committee. 24 h before neutron irradiation, human pancreatic cell lines (panc0403, PANC-1) were treated with 100 mM Glucose-BSH (final concentrations) or 100 mM BSH (final concentrations), or 100 mM BPA (final concentrations). Just before neutron irradiation, the cell samples were transferred to collecting tubes and subjected to 5 MW neutron irradiation (thermal neutron flux 1.4×10^9 neutron/cm²/s) for 15 min (thermal neutron fluence 4.4×10^{11} neutron/cm², γ-ray 0.7×10 Gy), 30 min (thermal neutron fluence 1.1×10^{12} neutron/cm², γ-ray 1.7×10^{-1}), or 45 min (thermal neutron fluence 1.1×10^{12} neutron/cm², γ-ray 1.7×10^{-1}).

After irradiation, all pancreatic cells were re-cultured in 96 well plates (9×10^3 cells/well) for 24 h and 48 h, and cell proliferation was measured with Cell Proliferation Reagent WST-1 (Roche, Basel) using a microplate reader (Vient XS, DS Pharma Promo Co., Ltd). A colony formation assay was carried out after 2 weeks of culture with Panc0403, PANC-1 in 60 mm culture dishes (n =4) and all culture cells were stained with 0.5% Crystal Violet (CV) in 20% methanol. The areas of colonies of CV stained samples were automatically calculated using a hybrid cell count application (BZ-H4C, KEYENCE, Osaka, Japan) in the BZ-X Analyzer software (BZ-H4A, KEYENCE, Osaka, Japan).

Neutron irradiation experiments on mouse models of subcutaneous tumors at

KURNS

Mice transplanted with Panc0403 CA19-9 producing cells were fixed in a mouse holder under trichloroanesthesia (medetomidine 0.3 mg/kg, midazolam 4 mg/kg, butorphanol 5 mg/kg). Twelve mice were fixed circumferentially to a 30 x 30 cm irradiation plate, and the irradiation plate was placed in a neutron irradiation box. There was a 12cm diameter circular hole in the center of the irradiation plate, and the outside of the circle was covered with a neutron shield made of enriched ^6LiF , making the outside the non-irradiated area and the inside the irradiated area. The position to fix the mouse was adjusted, and a subcutaneous tumor in the right lower abdomen was placed within the irradiated area. Neutrons from the Core were adjusted with a Spectrum shifter and irradiated into the irradiation box in which the mouse was fixed (Figure S8).

Dose estimation for BNCT

The total biological dose for BNCT consists of a nitrogen dose, hydrogen dose, gamma ray dose and boron dose. The nitrogen dose is originated from the $^{14}\text{N}(\text{n},\text{p})^{14}\text{C}$ reaction mainly for thermal neutron. The hydrogen dose is originated from the $^1\text{H}(\text{n},\text{n})^1\text{H}$ reaction mainly for fast neutron. The gamma ray dose is from the primary and secondary gamma rays. The secondary gamma ray is originated mainly from the $^1\text{H}(\text{n},\gamma)^2\text{D}$ reaction mainly for thermal neutron. The boron dose is originated from the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ reaction mainly for thermal neutron. Neutron fluence was measured by the activation foil method using gold foil. The physical neutron dose was calculated by multiplication of neutron fluence with kerma factor. The nitrogen and hydrogen doses were calculated by multiplication of the physical neutron doses with RBEs (relative biological effectiveness). The gamma ray dose was measured using a thermos-luminescence dosimeter. The boron dose was calculated by multiplication of the physical neutron dose with CBE and ^{10}B concentration. As described above, the final BNCT dose is expressed in grey-equivalents (Gy-Eq), which is the sum of the various physical dose components multiplied by the appropriate biological effectiveness factor.(Figure S1A). The RBE of neutrons was set to 3.0, which is the value normally used at the BNCT reactor neutron source in Kyoto University [38]. In this study, we did not calculate the CBE values for each organ for Glucose-BSH; since the intracellular uptake mechanism of Glucose-BSH is similar to that of BPA, the CBE values of BPA were used to estimate boron doses, but only predicted doses. The CBE values used were 2.5 for skin, 3.8 for tumor, 4.9 for duodenum (mucosa), and 4.25 for liver and pancreas as reference doses (Fig. 6D and 7I-N) [39].

CCLE

(<https://portals.broadinstitute.org/ccle>), we analyzed the expression of GLUT1, GLUT3, and LAT1 in pancreatic cancer cell lines for 8 of the 41 cell lines available for analysis. We compared the correlation of GLUT1, GLUT3, and LAT1 expression in pan-cancer and pancreatic cancer cell lines. The data was analyzed using Microsoft Excel to calculate Pearson correlation coefficient.

BNCT Simulation of dose–volume histogram to pancreatic cancer patient

The dose-planning system “SERA” was used to estimate dose distribution in body with previously simulation method[40]. Pharmacokinetic results were obtained from a mouse orthotopic model using PANC0403. Simulations were performed using pancreatic cancer patient data divided into non-open external irradiation data and open irradiation data. In the simulations, conditions were assumed to minimize damage to other organs, with a minimum tumor dose of 20 Gy-eq. The use of patient data was approved by the ethics committee of Okayama University and Okayama University Hospital (approval number: OU-research 2004-028).

Statistical analysis

Statistical analysis was conducted using Student’s *t* test, one-way analysis of variance (ANOVA), and two-way ANOVA with post hoc analysis. The *P* values less than 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism 9 software.

RESULTS

Synthesis of 1,2,3,4,5,6,7,8,9,10,11-Undecahydro-12-(1-Thio-β-D-Glucopyranose)-

Dodecaborate (G-BSH)

The synthesis of 1,2,3,4,5,6,7,8,9,10,11-undecahydro-12-(1-thio-β-D- glucopyranose)-dodecaborate (G-BSH) was outlined in Fig 1A. G-BSH was prepared in a two- step process: (a) conjugation of acetyl-protected glucopyranose unit with B₁₂-cluster core

and (b) deprotection of the acetyl protection groups of the Glucose-BSH (acetyl protection). Treatment of 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide with BSH in the presence of triethylamine in acetonitrile gave Glucose-BSH (Acetyl protection) in 19% yield. Finally, deprotection reaction of Glucose-BSH (Acetyl protection) with sodium methoxide gave the target G-BSH in 80% yield. The structure of G-BSH was confirmed by ^1H and ^{13}C NMR and IR spectroscopy as well as ESI-mass spectrometry measurements. The ^1H NMR spectrum of G-BSH shows all the expected resonances for the glucopyranose unit and boron cluster core (0.6-2.0 ppm) (Fig. 1B). Deprotection of all the acetyl groups of G-BSH (acetyl protection) was also supported by ^1H NMR spectrum and IR spectrum (Fig. 1C). After the deprotection, the methoxy proton signals of acetyl-group of G-BSH (acetyl protection) and the C=O stretching vibration at 1731 cm^{-1} of G-BSH (acetyl protection) disappeared. ESI-mass spectrometry measurements provided further evidence for the successful synthesis of G-BSH (Fig. 1D). The mass spectrum of G-BSH in positive-ion mode showed a molecular ion peak at $m/z = 395.2440$ $[\text{M}+\text{Na}]^+$; $\text{C}_6\text{H}_{22}^{10}\text{B}_{12}\text{Na}_3\text{O}_5\text{S}$ requires $m/z = 395.2433$). HPLC analysis confirmed the high purity of the G-BSH and we did not detect any of the compound.

Analysis of GLUT and LAT in CA19-9 producing and non-producing human PC cell lines

Using the publicly available Cancer Cell Line Encyclopedia (CCLE), the expression of boron drug target transporters was analyzed using Microsoft Excel to calculate Pearson correlation coefficient. No significant correlation was found between glucose transporter (GLUT) 1 and L-amino acid transporter-1 (LAT1) ($R=-0.009$) or between GLUT3 and LAT1 ($R=0.087$) in 947 cell lines and 36 cancer types (Fig. 2A, B). In contrast, GLUT1 and LAT1 ($R=-0.343$) and GLUT3 and LAT1 ($R=-0.245$) showed inverse correlations in human CA19-9 producing pancreatic cancer cells (Fig. 2C, D).

ELISA confirmed the secretion of CA19-9 into the culture medium of CA19-9 producing cells (BxPC3, S2-028, panc0504, panc0403) but not CA19-9 non-producing cell lines (PANC-1, QGP-1) (Fig. 2E). **CA19-9 localization in the cytoplasm of pancreatic cancer cell lines was confirmed by fluorescent cell immunostaining using anti-CA19-9 antibody** (Fig. 2F).

Western blotting of CA19-9-expressing pancreatic cancer cells (BxPC3, S2-028 and panc0504) revealed high GLUT1 and low LAT1; GLUT3 was only expressed in one of the CA19-9-expressing pancreatic cancer cell lines (panc0403) (Fig. 2G). The CA19-9 non-producing cells (PANC-1, QGP-1) showed high LAT1 expression but low GLUT1

and GLUT3 expression. Gene expression in the five cell lines using CCLE was consistent with the western blotting results (Fig. 2H).

CA19-9 producing PC cells showed specific high uptake of the novel boron drug

Glucose-BSH

The three boron drugs used in this study were Glucose-BSH (G-BSH), BSH, and BPA, with molecular weights of 372.43, 213.88, and 208.21, respectively, and intramolecular boron contents of 0.322, 0.561, and 0.048 ngB, respectively (Fig.1 and Fig. 3A).

We treated three pancreatic cell lines, BxPC3 and panc0403 CA19-9 producing cells and PANC-1 CA19-9 non-producing cells with the boron drugs and examined intracellular boron concentrations. All three cell lines, both CA19-9 producing and CA19-9 non-producing cells, showed low intracellular boron concentrations after treatment with BSH and BPA. In contrast, very high intracellular boron concentrations in the G-BSH group were observed in the BxPC3 and panc0403 CA19-9 producing cells, while low concentrations were observed in the PANC-1 CA19-9 non-producing cells (Fig. 3B). Cell immunostaining at the time after administration of Glucose-BSH into the culture medium showed intracellular uptake and diffusion in CA19-9 producing pancreatic cancer cells BxPC-3, panc0504, and panc0403 from 2 hours after

administration, and some of them were observed to be transported near the nuclear membrane and into the nucleus. The same results were observed at 6 and 12 hours post-dose. In contrast, in the CA19-9 non-producing pancreatic cancer cell PANC-1, only a little intracellular uptake was observed from 2 to 12 hours after Glucose-BSH administration(Fig. 3C).

We then performed similar experiments as in Fig. 3B; the boron content of the administered drugs was matched and intracellular boron uptake was evaluated. The results showed that high specific uptake of G-BSH was observed in CA19-9 producing PC cells but not in CA19-9 non-producing PC cells (Fig. 3D).

We evaluated the cytotoxicity of Glucose-BSH on three PC cell lines (BxPC3, panc0403, PANC-1) and mouse fibroblasts. No apparent toxicity was observed after 48 h of treatment (Figure S2).

Glucose-BSH uptake into CA19-9 producing pancreatic cancer cells is enhanced in a low glucose environment and blocked by glucose transporter inhibition.

To exclude the possibility that cell death was from glucose depletion, we cultured cell lines under varying glucose concentrations (0–2.0 g/L) (Fig. 4A). We then cultured cells with three levels of glucose concentration in cell culture (0.5 g/L in the low glucose

group, 2 g/L in the control group, and 4.5 g/L in the high glucose group) and exposed cells to G-BSH, BSH, and BPA to measure boron concentrations. In CA19-9 producing pancreatic cancer cells, Glucose-BSH uptake was enhanced in a low glucose environment, while BPA uptake was unaffected; in CA19-9 non-producing pancreatic cancer cells, there was no change in uptake of either Glucose-BSH or BPA in a low glucose environment (Fig. 4B). These results indicate that in CA19-9 producing pancreatic cancer cells, Glucose-BSH uptake is higher under low glucose conditions. Western blotting was then performed in cells cultured in various glucose concentrations. In CA19-9 producing pancreatic cancer cells, the expressions of GLUT1 and GLUT3 increased with decreasing glucose concentration; in CA19-9 non-producing pancreatic cancer cells, no changes in GLUT1 and GLUT3 expression were observed (Fig. 4C). We next treated cells with a GLUT inhibitor (WZB117) and measured viability and Glucose-BSH uptake. WZB117 concentrations below 10 μ M for BxPC3 cells and below 20 μ M for panc0403 cells had no effect on survival; these concentrations were thus selected as experimental concentrations (Figure S3A, B). We then treated cells with WZB117, followed by Glucose-BSH, and measured boron concentrations. In both BxPC3 and panc0403 CA19-9 producing pancreatic cancer cells, boron concentrations were decreased with increasing amounts of WZB117 (Fig. 4D). The 10 μ M GLUT

inhibitor treatment resulted in 4.3 ± 0.1 and 7.7 ± 0.3 inhibition of intracellular boron concentrations in BxPC3 and panc0403 cells. These results indicated that Glucose-BSH uptake is mostly regulated by GLUT.

Glucose-BSH showed the highest efficacy after neutron irradiation of CA19-9 producing pancreatic cancer cells.

We next performed colony formation assays in CA19-9 producing PC cells panc0403 and CA19-9 non-producing cells PANC-1 treated with boron agents and irradiated with neutrons. In panc0403 cells, Glucose-BSH showed superior inhibition of cell proliferation compared with the Control, BPA, and BSH treatments starting from 30 min of irradiation and 45 min ($P > 0.001$). In contrast, none of the boron agents showed significant cell-killing effects in CA19-9 non-producing PANC-1 cells (BPA: $P > 0.4$, BSH: $P > 0.6$) (Fig. 5A). Treatment doses were as high as 19.1, 34.9, and 53.2 Gy-Eq each in the Glucose-BSH group of panc0403 cells for 15, 30, and 45 min of irradiation, respectively; the Glucose-BSH group showed 5-fold higher neutron doses than the BPA and BSH groups (Fig. 5B and Figure S4). These results indicate that Glucose-BSH showed strong cytotoxic activity against CA19-9 producing pancreatic cancer cells upon neutron irradiation.

Pharmacokinetics of Glucose-BSH and anti-tumor effects in a subcutaneous pancreatic cancer mouse model.

We then examined the potential anti-tumor effects of G-BSH in vivo. CA19-9 producing cells panc0403 were transplanted into the right side of the abdomen of BALB/c-nu/nu mice to establish the pancreatic mouse model, and Glucose-BSH (75 mg/kg) was administered intraperitoneally or subcutaneously. Tumor and other tissues (blood, liver, brain, kidney, brain, muscle, skin, pancreas, and small intestine) were collected 0.5–12 h after administration for pharmacokinetic evaluation by ICP-MS (n=4, each tissue type).

In the intraperitoneal treatment group, the highest intra-tumor boron concentration was 23.8 ppm¹⁰B at 0.5h after administration, and boron concentrations in blood, skin, and pancreas were 11.1, 7.7 and 4.1 ppm¹⁰B, respectively (Fig. 6A), with high Tumor/Blood and Tumor/Normal tissue ratios (T/B: 2.2, T/N(skin): 3.1, T/N(pancreas): 5.8) (Fig. 6B).

In the subcutaneous treatment group, the highest intra-tumor boron concentration was 13.8 ppm¹⁰B at 1 h after administration (Figure S6A), and the T/B and T/N ratios were also lower (T/B ratio: 1.3, T/N(skin): 2.4, T/N(pancreas): 3.2) (Figure. S6B).

From these results, neutron irradiation was performed 0.5 h after 75 mg/kg intraperitoneal administration of boron drugs on day 7 after transplantation. Tumor

volumes were measured 34 days after irradiation (41 days after transplantation) in the Control, BPA, BSH and G-BSH groups (n=6 each): 779.0 ± 166.2 , 336.2 ± 144.4 , 412.8 ± 83.5 and $14.1 \pm 6.7 \text{ mm}^3$, respectively (Fig. 6C). Treatment doses were 2.3 ± 0.2 , 7.6 ± 0.9 , 5.7 ± 0.6 and $31.7 \pm 5.1 \text{ Gy-Eq}$ in Control, BPA, BSH and G-BSH groups (Figure S1A,B,C and Fig. 6D).

Although many studies using glucose-bound drugs have been reported, some drug development has been discontinued due to problems with neurotoxicity caused by drug accumulation in the brain. In our experiments, brain boron concentrations after intraperitoneal administration of glucose-BSH were 0.3, 0.3, 0.3, 0.2, and 0.1 ppm^{10B} from 0.5 to 12 hr, with little brain transport observed (Fig. 6A). In addition, the Papp coefficient of Glucose-BSH was 0.2 in the blood-brain barrier crossing test using the BBB Kit, indicating that Permeability was very low (Figure S5).

Pharmacokinetics of glucose-BSH in an orthotopic mouse model of pancreatic cancer and dosimetry in boron neutron capture therapy for pancreatic cancer

CA19-9 producing cells panc0403 were then transplanted under the anterior pancreatic capsule of BALB/c-nu/nu mice to create a pancreatic orthotopic mouse model, and Glucose-BSH (75 mg/kg) was administered intraperitoneally (Fig. 7A, B, C). The

method of drug administration was based on previous studies [\[\[41\]\]](#), and we chose a method other than the intravenous injection protocol because intravenous anesthesia may affect the circulation, blood flow, and tissue distribution of the boron drug. While previous studies used a subcutaneous injection protocol for BPA, we chose the intraperitoneal protocol for Glucose-BSH because intraperitoneal administration resulted in higher accumulation of the boron drug in the tumor than in subcutaneous administration experiments (Fig.6A, Figure. S6) and a better T/N ratio. Tumors and other tissues (blood, pancreas, stomach, duodenum, liver, colon, spleen, kidney, and skin) were collected 0.5-4 h after administration for pharmacokinetic evaluation by ICP-MS (n=4, each tissue type). One hour after administration, the boron concentration in blood, skin, and pancreas was 12.1, 3.9, and 2.3 ppm¹⁰B, respectively (Fig. 7D), with high tumor/blood and tumor/normal tissue ratios (T/B: 2.1, T/N (skin): 6.5, T/N (pancreas): 10.8) (Fig. 7E).

Assuming a minimum tumor dose of 20 Gy-eq, BNCT treatment simulations were performed for pancreatic head and tail cancers when Glucose-BSH (75 mg/kg) was administered, and dose volume histograms (DVH) were shown (Fig. 7F, G, H, I, J, and K). As optimization results, external irradiation of the pancreatic head cancer required two irradiations (Fig. 7F). The mean and maximum doses were, respectively, tumor

(23.9, 28.5 Gy-eq), normal pancreas (6.4, 7.9 Gy-eq), and liver (29.7, 105 Gy-eq) (Fig. 7I, L). Since the dose to the liver is very high in the treatment of pancreatic head cancer by external neutron irradiation, the simulation was performed with open neutron irradiation. Laparotomy was performed, and part of the liver, part of colon, and whole stomach located ventral to the pancreas were fixed outside the irradiation area to simulate open single portal irradiation of the pancreatic head cancer from the front of the pancreas (Fig. 7G). Assuming a rectangular irradiation field of 80mm in length and 200 mm in width so that the heart and lungs do not enter the irradiation field, the mean and maximum doses were 21.7 and 22.4 Gy-eq for the tumor, 3.4 and 4.4 Gy-eq for the normal pancreas, and 14.9 and 42.2 Gy-eq for the liver, respectively (Fig. 7J, M). In the case of one portal external irradiation for pancreatic tail cancer from 15 degrees behind the left, the mean and maximum doses were as follows: tumor (27.2, 37.7 Gy-eq), normal pancreas (2.5, 7.6 Gy-eq), and liver (2.4, 73.8 Gy-eq), respectively (Fig. 7H, K, N). Glucose-BSH pharmacokinetic results using an orthotopic model of pancreatic cancer and BNCT simulation data for pancreatic head and tail carcinoma showed that Glucose-BSH BNCT results are promising for future clinical applications.

Results Summary of and future clinical application image of G-BSH-BNCT

GLUT is highly expressed and LAT1 expression is low in CA19-9 producing pancreatic cancer cells. The opposite is true for CA19-9 non-producing pancreatic cancer cells. In this experiment, we performed BNCT in a subcutaneous mouse model transplanted with CA19-9 producing pancreatic cancer cells in conjunction with tumor gene analysis using Glucose-BSH to induce tumor-specific cell-killing effects. Such BNCT tailored to tumor gene analysis is called precision BNCT (Fig. 8A). The CA19-9 level greatly influences the indication for surgery and prognosis in localized pancreatic cancer. Preoperative BNCT (external or open irradiation) can be used to control CA19-9 in pancreatic cancer patients with high CA19-9 levels even after NAC, and more patients can be treated with radical surgical resection (Fig. 8B).

Discussion

The most important aspect of BNCT therapy is the selective uptake of the boron drug by tumor cells and the selective killing of tumor cells by neutron irradiation through nuclear reactions between boron and neutrons[42-44].

Currently, the only boron agent used in clinical practice for BNCT is the amino acid derivative BPA, which is very useful for the current clinical indication of head and neck cancer[14, 45]. However, BPA is limited to tumors that express high levels of LAT1.

Our analysis showed that pancreatic cancer is a tumor with low LAT1 expression across cancer types in The Cancer Genome Atlas (Figure S7). Therefore, boron agents that accumulate in pancreatic cancer are needed for BNCT of pancreatic cancer. In this study, we found that malignant pancreatic cancers expressing high CA19-9 levels express low levels of LAT1, while malignant pancreatic cancers expressing low CA19-9 levels express high levels of LAT1 (Fig. 2I), indicating that BPA is not effective for high CA19-9 malignant pancreatic cancers.

Pancreatic cancer tumors are rich in stromal content, with poor angiogenesis and pathology and poor contrast effect on imaging studies[45, 46]. The poor reach of low molecular weight contrast agents in pancreatic cancer indicates that it is a tumor with poor enhanced permeability and retention (EPR) effect from a drug delivery system perspective[47]. While various polymeric drugs, including liposome formulations, have been developed in boron drug research, these are tumor-selective drugs based on EPRs, and their clinical application to pancreatic cancer was considered difficult[37, 48, 49].

We focused on the fact that pancreatic cancer is diagnosed by ^{18}F -FDG-PET and designed a boron drug with glucose binding, which is a basic component of FDG. We found that the expression of GLUT correlates with the standardized uptake value of ^{18}F -FDG-PET accumulation in pancreatic cancer. Additionally, studies showed that

blood CA19-9 correlates with standardized uptake value maximum (SUV max) in pancreatic cancer[50, 51]. Therefore, we developed G-BSH based on the idea that a glucose-binding boron drug could be specifically incorporated into pancreatic cancers with high levels of CA19-9. Previous studies reported that glucose concentrations in the tumor microenvironment are lower than those in non-tumor areas because tumor areas require more sugar for cell proliferation[52]. G-BSH showed more enhanced cellular uptake in a low glucose environment (Fig. 4B), making G-BSH unparalleled in its ability to achieve tumor-selective hyperaccumulation independent of EPR effects.

Various studies have been conducted on glucose-binding drugs. Although there have been many examples of successful drug binding to glucose positions 1, 2, and 6, there have been no reports of successful binding to positions 3 and 4[33, 53]. A typical example is the anticancer drug ifosfamide bound to glucose, which underwent phase II clinical trials but was withdrawn from use because of brain toxicity[54]. Glucose-BSH is a new drug that binds to BSH at the glucose-1 position (Fig. 1A) and is safe without cytotoxicity (Figure S2). Our results also indicate that it may not have side effects as a result of brain transit because it has no BBB transit ability (Fig. 5).

In recent years, the diversity of cancer cells from intra-tumor genetic polymorphisms has been investigated. Malignant tumors are composed of a number of subclones and

simultaneous targeted therapy of multiple molecules in tumors can improve therapeutic efficacy[55]. BNCT has no effect on subclones without boron uptake. Our results showed that Glucose-BSH is selectively taken up via GLUT in high CA19-9 pancreatic cancer. Therefore, it may be possible to target pancreatic cancer subclones with low CA19-9 levels in addition to pancreatic cancer subclones with high CA19-9 levels by combining this drug with BPA, which is taken up via LAT1; this approach may achieve even higher therapeutic efficacy.

BNCT clinical trials for pancreatic cancer have not yet been developed worldwide, and careful irradiation methods need to be considered for realization. In particular, BSH has been shown to be highly concentrated in the liver, and BNCT to the liver was tested in a clinical trial in 2006, where irradiation and reimplantation were performed after liver resection to minimize the extent of irradiation[56]. Our Glucose-BSH was also expected to be highly concentrated, and indeed boron pharmacokinetic measurements after Glucose-BSH administration showed high liver uptake (Fig. 8I). The irradiation range and direction were set to minimize damage to surrounding organs, and open irradiation was simulated assuming a minimum tumor dose of 20 Gy-eq. External irradiation of the pancreatic head required irradiation from two directions, and although the dose to the surrounding organs including the liver was higher, it was possible to

secure the tumor dose. Assuming that the irradiation area of neutron irradiation is about 200 mm in width, a part of the left lobe of the liver is expected to be included in the irradiation field in actual clinical external irradiation of pancreatic head cancer, but the minimum remaining liver volume after surgical resection is reported to be 20% for normal liver and about 40% for cirrhotic liver, and irradiation is not fatal in the case of liver left lobe failure possibility[57]. One of the greatest advantages of open irradiation of the pancreatic head is that it allows selective high dose to the tumor, and the liver, part of the large intestine, and the stomach, which are located ventral to the pancreas, can be fixed outside the irradiated area. In the case of irradiation of pancreatic tail cancer, it was possible to secure a tumor dose with a single portal irradiation by arranging the irradiation direction.

In the dose simulations, since there are no previous reports of CBE in the pancreas and duodenum, the same values as BPA were used for skin 2.5 and tumor 3.8 for the organs with previous reports, since Glucose-BSH shows intracellular boron uptake comparable to that of BPA. For the duodenum, which has not been previously reported, the reference dose was 4.9 for oral mucosa, which is the same glandular cell, and for the pancreas, 4.25 for liver (Fig. 6A and 7A) [39].

Early detection of pancreatic cancer and surgery for resectable pancreatic cancer are

the mainstays of the current curative pancreatic cancer treatment[58]. However, after rigorous preoperative evaluation, including blood CA19-9 levels, surgery is performed in only 10%–20% of patients with pancreatic cancer[59]. Among the resectable pancreatic cancers, 49% have elevated CA19-9 levels, and even among stage IA cases, 38% are CA19-9-positive[25, 60]. Therefore, for resectable pancreatic cancer, it is most important to control CA19-9 levels within the reference range before surgery; for pancreatic cancer with high CA19-9 levels, it is necessary to lower the blood CA19-9 level to make the cancer operable[61]. The relationships of CA19-9 with carcinogenesis or cancer progression have been somewhat unclear. However, some studies have shown that CA19-9 functions in the development and progression of pancreatitis and pancreatic cancer[30]. Therefore, in recent years, CA19-9 antibody therapies that directly target CA19-9 (HuMab-5B1, MVT-5873) and ¹⁷⁷Lu human monoclonal antibody 5B1 (MVT-1075) in combination with a blocking dose of MVT -5873 as radioimmunotherapy have been developed, and ⁸⁹Zr-labeled anti-CA19-9 antibody imaging clinical trials are underway; research studies targeting CA19-9 are also very active[62-64].

We speculate that the combination of these CA19-9-targeted therapies with Glucose-BSH-BNCT may represent a new direction toward the cure of pancreatic

cancer. In this study, we analyzed the gene expression of pancreatic cancers showing increased glucose uptake and confirmed the antitumor effect of neutron irradiation on pancreatic cancers with high CA19-9 levels by using a new boron drug that binds BSH to glucose. Recently, various studies have been conducted on CA19-9 itself as a therapeutic target[65], and we are now working on the binding of boron agents to sugar compounds, a component of CA19-9, and BNCT using a novel boron agent based on the method of this study as our next research project. BNCT has shown success in basic research and clinical BNCT in head and neck cancer, skin malignancies, etc. On the other hand, less significant results have been reported in deeper tumors such as pancreatic cancer. On the other hand, there have been few reports of significant results in deep-seated tumors such as pancreatic cancer. This is because (1) drugs such as BPA are not taken up specifically in pancreatic cancer, (2) the EPR effect is low and polymeric drugs are difficult to reach, and (3) neutron irradiation is difficult even using extra-thermal neutrons if the tumor is deeper than 6 cm below the surface. In this study, we proposed a project called Precision BNCT, which combines genetic analysis and BNCT boron drug discovery. We proposed the efficacy of glucose-BSH formulation for pancreatic cancer with high CA19-9 levels. Furthermore, in deep cases, especially in pancreatic cancer, we proposed neutron irradiation by laparotomy, and furthermore, we

showed that irradiation avoiding the liver is sufficient to deal with deep organs (pancreatic cancer). In this context, it is important to increase the number of drug variations, to select drugs according to cancer and genetic analysis, and to show new BNCT therapies such as open abdominal irradiation. The results of this experiment showed that both of these possibilities are sufficient for the extension of the current BNCT" I included the above in the discussion. Thank you for your valuable comments.

We propose preoperative Glucose-BSH-BNCT as a new option for the multidisciplinary treatment of localized pancreatic cancer targeting CA19-9, which can lead to radical surgical resection along with preoperative chemotherapy, and contribute to the improvement of prognosis.

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Data Availability Statement

The article includes all data in the study, and further inquiries can be directed to the corresponding author.

Conflict of Interest

All authors have no conflict of interest in this project.

Credit Author Statement

Takuya Fujimoto: Investigation, Resources, Writing - Original Draft, Visualization, Formal analysis, **Fuminori Teraishi :** Methodology, Funding acquisition, Supervision and Funding acquisition, **Noriyuki Kanehira:** Investigation, Resources and software, Funding acquisition, **Tomoyuki Tajima :** Investigation, Methodology, Resources, and Writing - Original Draft, **Yoshinori Sakurai:** Software, Formal analysis and Methodology, Writing – original draft **Natsuko Kondo, Masahiro Yamagami, Atsushi Kuwada, Akira Morihara, Mizuki Kitamatsu, and Atsushi Fujimura:** Investigation and Resources, **Minoru Suzuki, Yutaka Takaguchi, Kunitoshi Shigeyasu, and Toshiyoshi Fujiwara:** Supervision, **Hiroyuki Michiue:** Conceptualization, Methodology, Writing- Reviewing, Project administration and Funding acquisition,

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Figure Legends

Fig. 1 Synthesis of a new glucose-bound boron drug (Glucose-BSH).

(A) Synthesis of a new glucose-bound boron drug (Glucose-BSH). (B) ^1H NMR spectrum of Actylated Glucose-BSH in D_2O . (C) Fourier transform infrared spectroscopy (FT-IR) spectrum of Actylated Glucose-BSH. (D) Electrospray ionization mass spectrometry (ESI-MS) spectrum in positive mode of Actylated Glucose-BSH. (E) The magnified view of ESI-MS of Actylated Glucose-BSH.

Fig. 2 Glucose transporter expression analysis in CA19-9 producing and non-producing human pancreatic cancer cells.

(A,B) mRNA expression analysis on the basis of RNA-seq data of LAT1 and GLUT1 (A) and LAT1 and GLUT3 (B) in human pan-cancer cell lines (1076 lines) using Cancer Cell Line Encyclopedia (CCLE) (A: $R=-0.009$, B: $R=0.087$) (C,D) mRNA expression analysis of LAT1 and GLUT1 (C) and LAT1 and GLUT3 (D) in human pancreatic cancer cells (seven lines) using CCLE on the basis of RNA-seq data (C: $R=-0.343$, D: $R=0.245$) (E) Comparison of GLUT1 expression in CA19-9-secreting and -non-secreting pancreatic cancer cells ($P=0.051$) (F), Evaluation of CA19-9 secretion into the culture medium of human pancreatic cancer cells by ELISA (G) Evaluation of CA19-9 production in the cytoplasm by fluorescence immunostaining (red: CA19-9, blue: nuclei) (H) Analysis of GLUT1/3 and LAT1/2 protein expression in CA19-9 producing human pancreatic cancer cells and CA19-9 non-producing pancreatic cancer cells (I) Analysis of GLUT1/3 and LAT1/2 gene expression in CA19-9 producing and CA19-9 non-producing human pancreatic cancer cells using CCLE

Fig. 3 Evaluation of cellular uptake of Glucose-BSH, a novel boron drug.

(A) Chemical structure of Glucose-BSH, a new boron agent, and existing boron agents BSH and BPA. (B) Glucose-BSH, BSH, and BPA were administered at $100\text{ }\mu\text{M}$ in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells, and the intracellular boron concentration was measured 24 h later using ICP (red: Glucose-BSH, green: BSH, blue: BPA). (C) Glucose-BSH $100\text{ }\mu\text{M}$ was administered in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells (four lines) and intracellular localization and uptake were observed using fluorescent immunostaining at 2, 6, and 12 h after administration (red: BSH, green: phalloidin, blue: nuclei). (D,E) CA19-9 producing and non-producing human pancreatic cancer cells were treated with boron drugs at matched B10 concentrations

(Glucose-BSH 50, 100 μ M, BSH 50, 100 μ M, BPA600, 1200 μ M) and 24 h later, the intracellular boron concentration was measured using ICP (red: Glucose-BSH, green: BSH, blue: BPA).

Fig. 4 Evaluation of glucose-BSH cellular uptake in response to changes in glucose concentration and inhibition of uptake by glucose inhibitors (WZB117) in a simulated cancer microenvironment

(A) CA19-9 producing and non-producing human pancreatic cancer cells were incubated with varying glucose concentrations (0, 0.25, 0.5, 0.75, 1.0, 2.0 g/L) and cell viability was measured after 24 and 48 h. Cell viability was confirmed even at a glucose concentration of 0.25 g/L. (B) CA19-9 producing and non-producing human pancreatic cancer cells were incubated with varying glucose concentrations (0.5, 2, 4.5 g/L) and the intracellular boron concentration was measured using ICP after 24 h. (C) Glucose concentrations in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells were varied (0.5, 2, 4.5 g/L), and protein expression of GLUT1, GLUT3, and LAT1 was determined after 36 h. (D) CA19-9 producing human pancreatic cancer cells were cultured in different concentrations of WZB117 (0, 1, 5, 10 μ M) for 24 h and then incubated with 100 μ M of Glucose-BSH for 24 h. The intracellular boron concentration was measured using ICP.

Fig. 5 Cell-killing effect of BNCT with Glucose-BSH in human pancreatic cancer cells.

(A) Panc0403 cells (CA19-9 producing human pancreatic cancer cells) and PANC-1 cells (CA19-9 non-producing human pancreatic cancer cells) were treated with 100 μ M of each boron drug (BPA, BSH, Glucose-BSH) for 24 h and then irradiated with neutrons. After irradiation, cells were cultured for 14 days and subjected to colony formation assay. (B) The graphs show the irradiation doses in panc0403 and PANC-1 cells treated with boron drugs (BPA, BSH, and Glucose-BSH).

Fig. 6 Anti-tumor effect of BNCT with Glucose-BSH on a pancreatic cancer in vivo model.

(A) Panc0403 cells (CA19-9 producing human pancreatic cancer cells) were transplanted into the right-side abdomen of mice to create a pancreatic cancer subcutaneous tumor model. Glucose-BSH (75 mg/kg) was administered intraperitoneally. Uptake concentration in organs was measured using ICP. (B) The tumor to normal ratio (T/N ratio) (blue: skin, yellow: pancreas) and tumor to blood ratio

(T/B) (red) were determined at various time points (0.5, 1, 2, 3, 6, 12 h) after intraperitoneal administration of Glucose-BSH in the subcutaneous tumor model of pancreatic cancer. (C) A panc0403 pancreatic carcinoma subcutaneous tumor model was created and 75 mg/kg of BPA, BSH, or Glucose-BSH was administered intraperitoneally on day 7 after transplantation. Neutron irradiation was performed at KURRI 1 h after administration, and tumor volume was monitored. (D) Total biological dose (Gy-Eq) from neutron irradiation in the control and boron-drug-treated groups (n=6). (E) Representative subcutaneous tumors in the control and boron drug groups on day 35 after neutron irradiation are shown.

Fig. 7 Mouse orthotopic model of pancreatic cancer and results of preclinical studies of neutron irradiation of pancreatic cancer patients

(A) Photograph of an orthotopic model of pancreatic cancer using the Panc0403 cell line (B) HE-stained histopathology results of pancreatic cancer sites (scale bar 500 μ m) (C) Fluorescent immunostaining using HLA class I (GFP) and nuclear staining (blue) in pancreatic cancer area (scale bar 500 μ m) (D) ICP results of boron pharmacokinetics in tumor and various organs of mouse pancreatic cancer orthotopic model after Glucose-BSH intraperitoneally administration (75 mg/kg) (0.5, 1, 2, 4h). (E) The tumor to normal ratio (T/N ratio) and tumor to blood ratio (T/B) (red) in mouse pancreatic cancer orthotopic model (0.5, 1, 2, 4 h). (F, G, H) Images of irradiation of pancreatic cancer patients with Glucose-BSH BNCT (F: two external neutron irradiation of pancreatic head cancer, G: open irradiation of pancreatic head cancer, H: external neutron irradiation of lateral abdomen of pancreatic tail cancer). (I, J, K, L, M, N) Glucose-BSH BNCT dose volume histogram (DVH) and BNCT maximum and average doses (I, L: two external neutron irradiation of pancreatic head cancer, J, M: open irradiation of pancreatic head cancer, H, K: lateral abdominal external neutron irradiation of pancreatic tail cancer) Simulation with a minimum tumor dose of 20 Gy-Eq.

Fig. 8

Abstract of this experiment and image of clinical application of BNCT using Glucose-BSH

(A) CA19-9 producing pancreatic cancer cells express more GLUT and CA19-9 non-producing pancreatic cancer cells express less GLUT. A subcutaneous tumor model was created by transplanting CA19-9 producing pancreatic cancer cells into BALB-c/nu-nu mice and using Glucose-BSH BNCT showed potent anti-tumor effects.

(B) Based on the results of this experiment, we believe that it will be possible to control CA19-9 levels by preoperative BNCT (external or open irradiation) for patients with localized pancreatic cancer who have high preoperative blood CA19-9 levels and are not eligible for radical surgery, and to make more patients eligible for second-stage radical surgical resection.

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Fig. 1

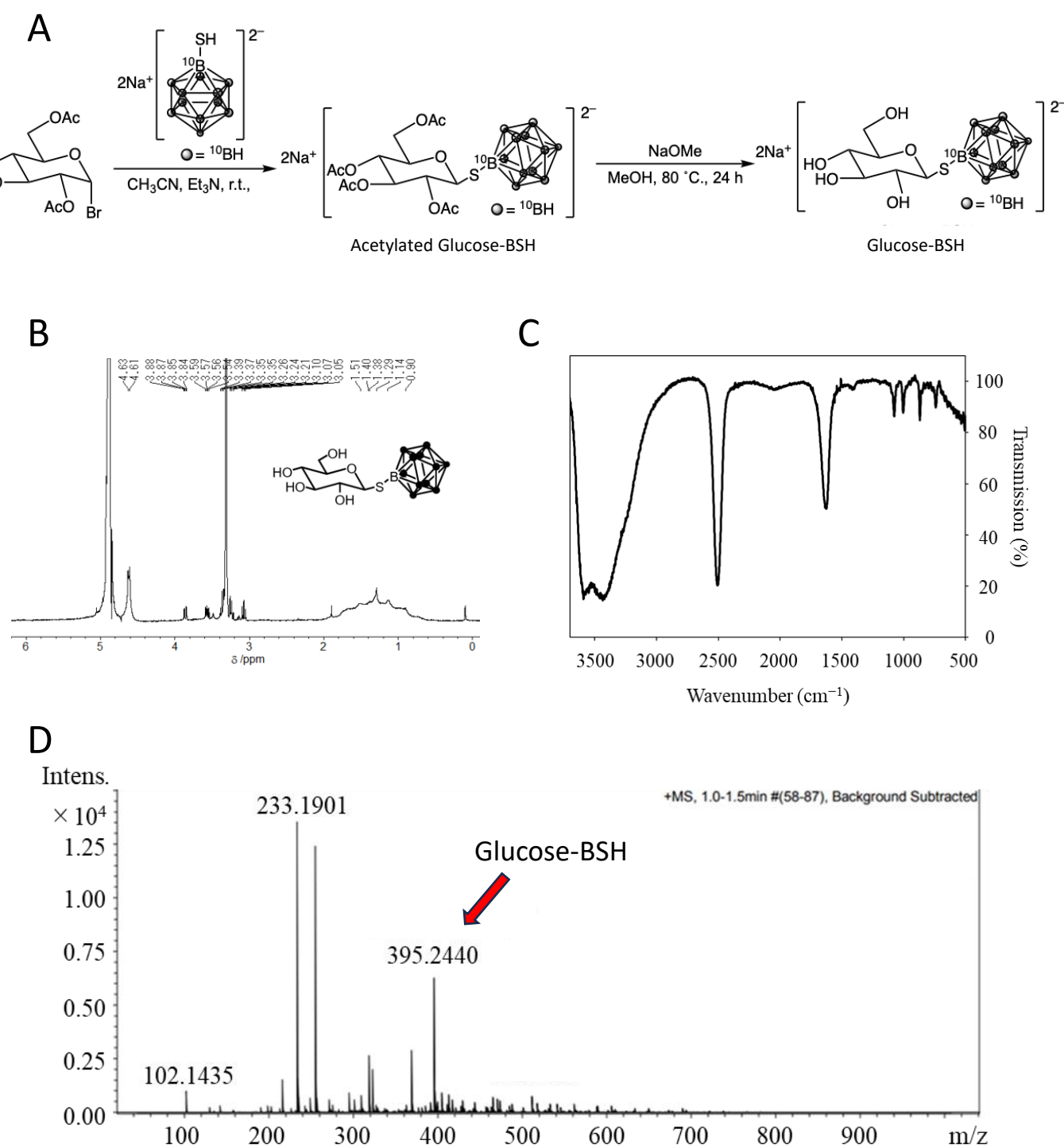


Fig. 2

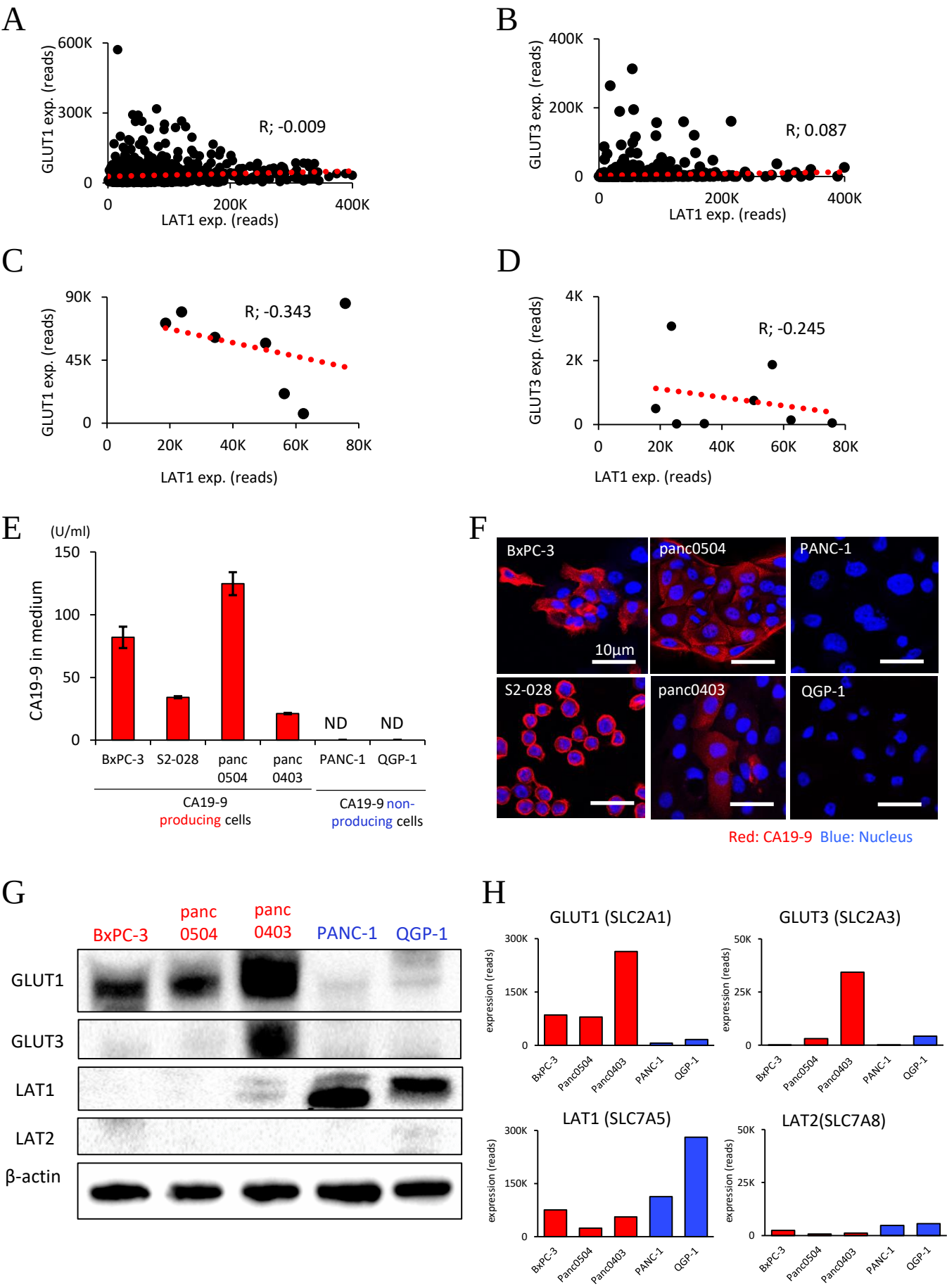


Fig. 3

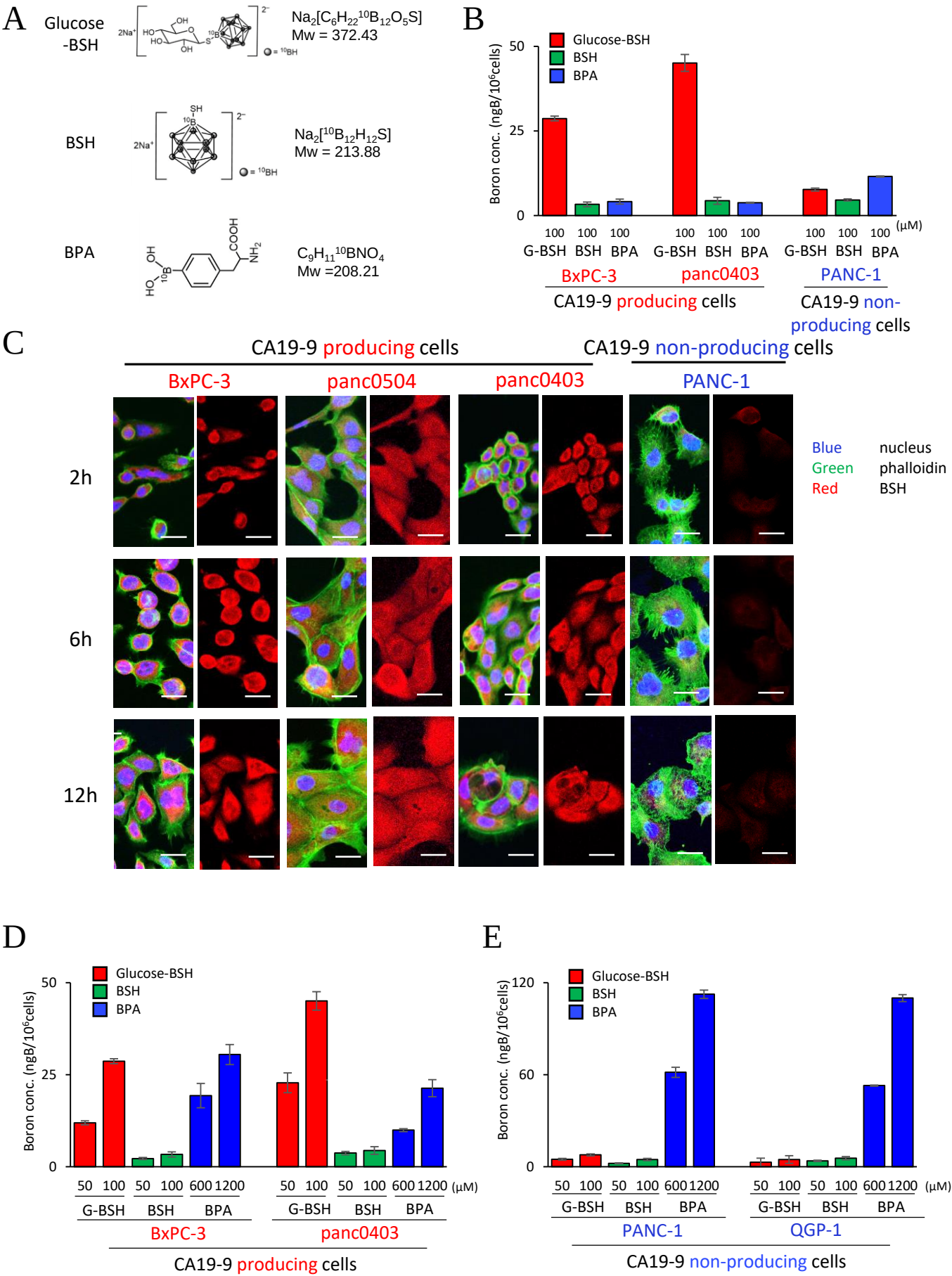


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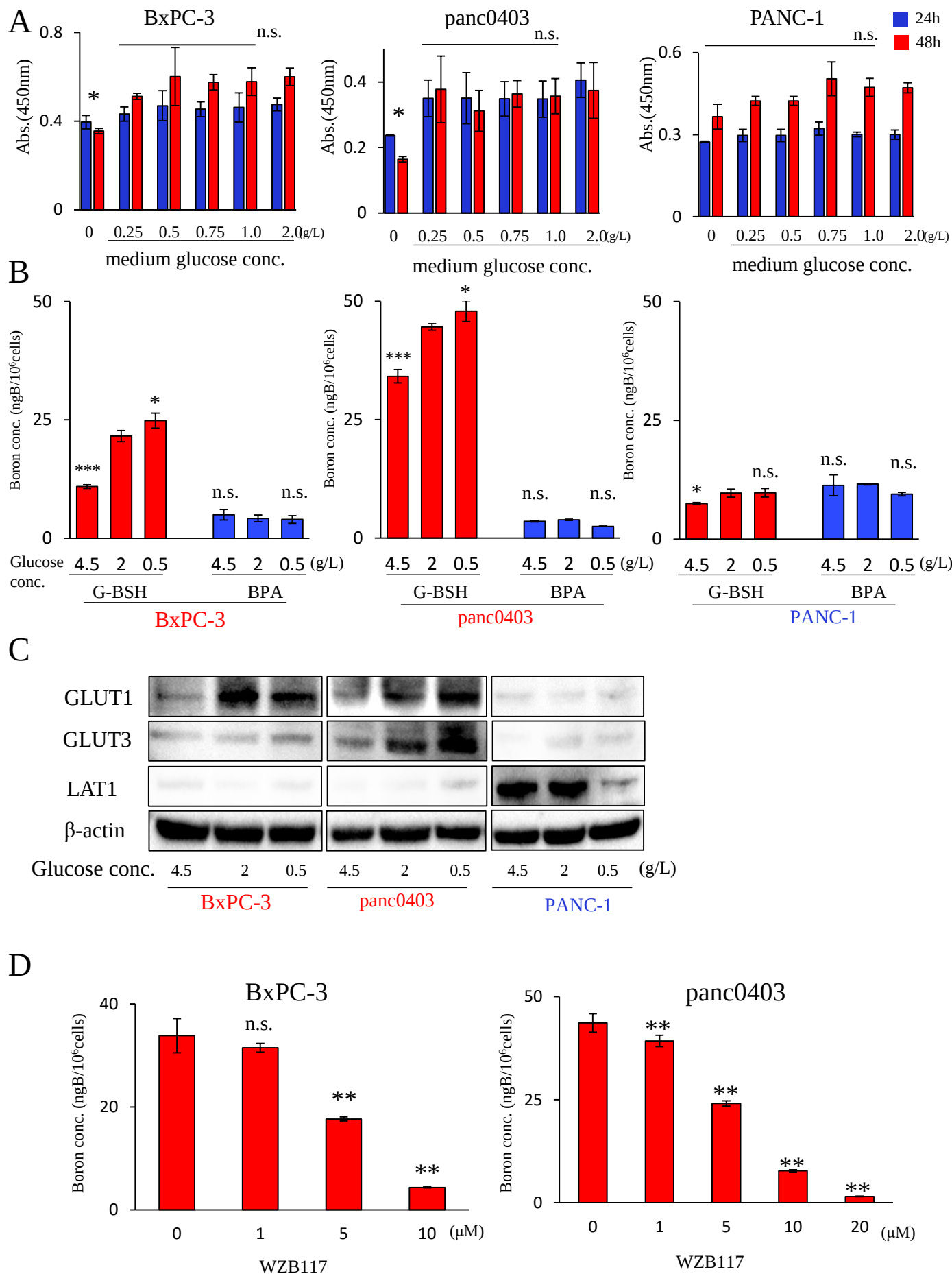


Fig. 5

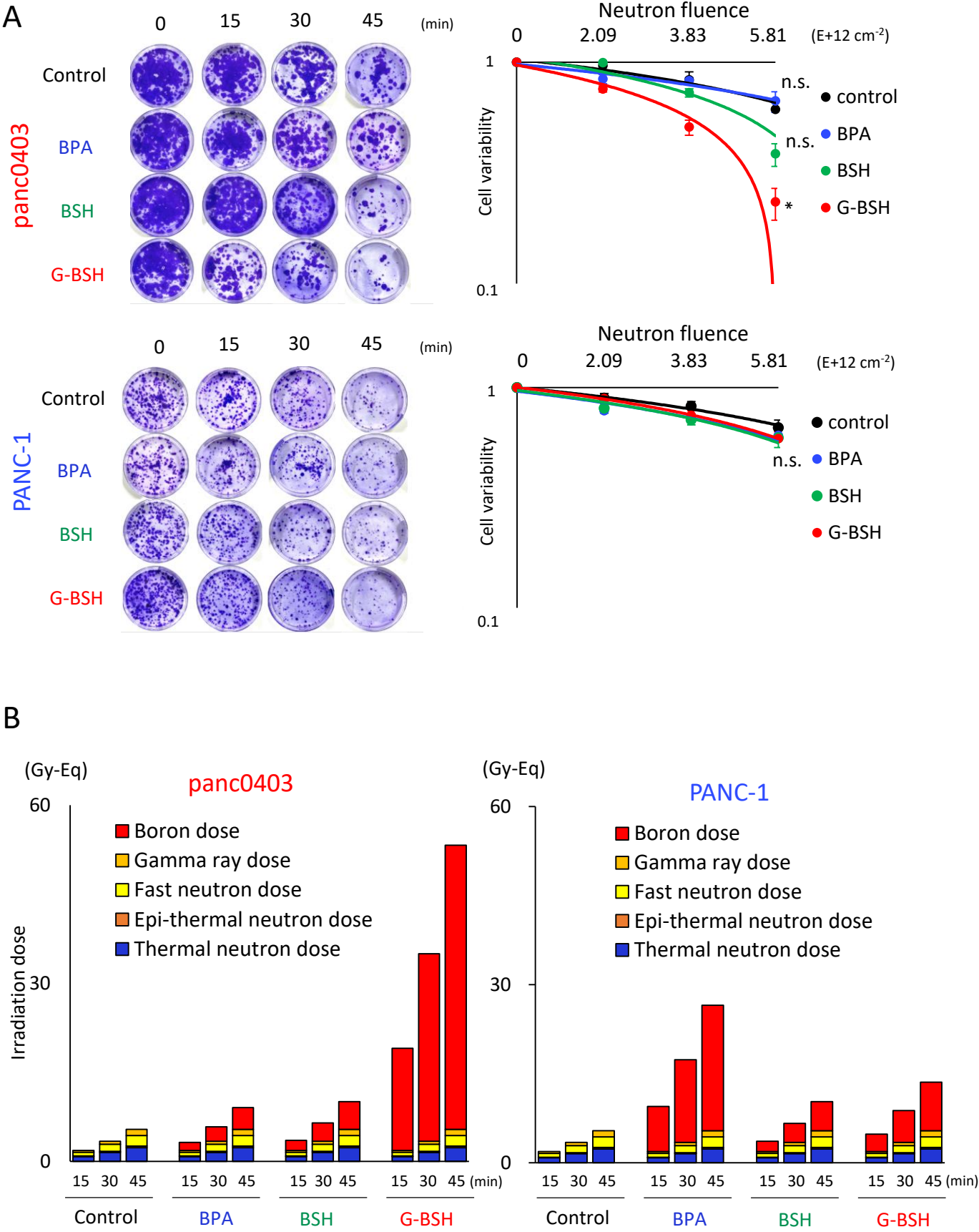


Fig. 6

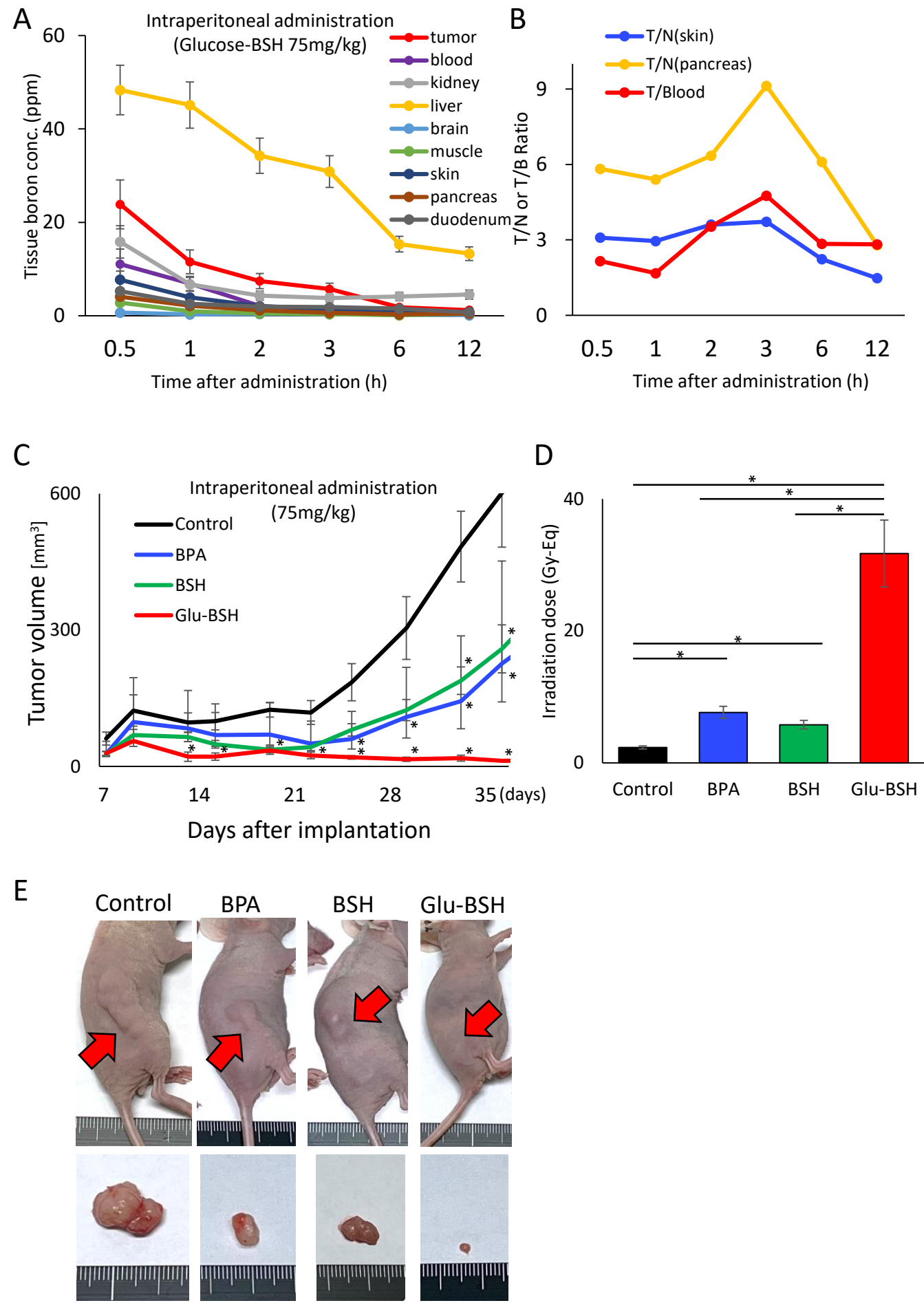


Fig. 7

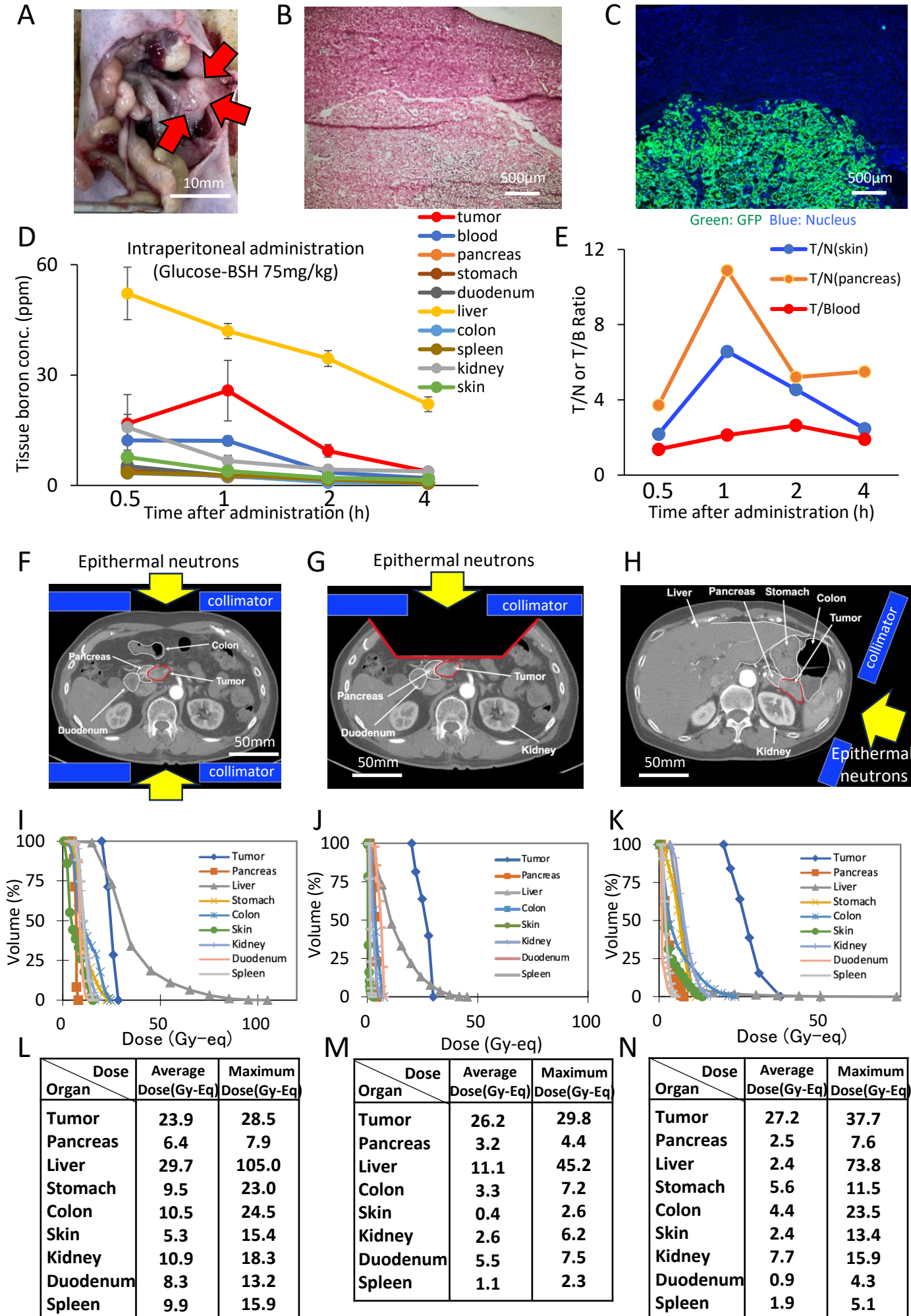
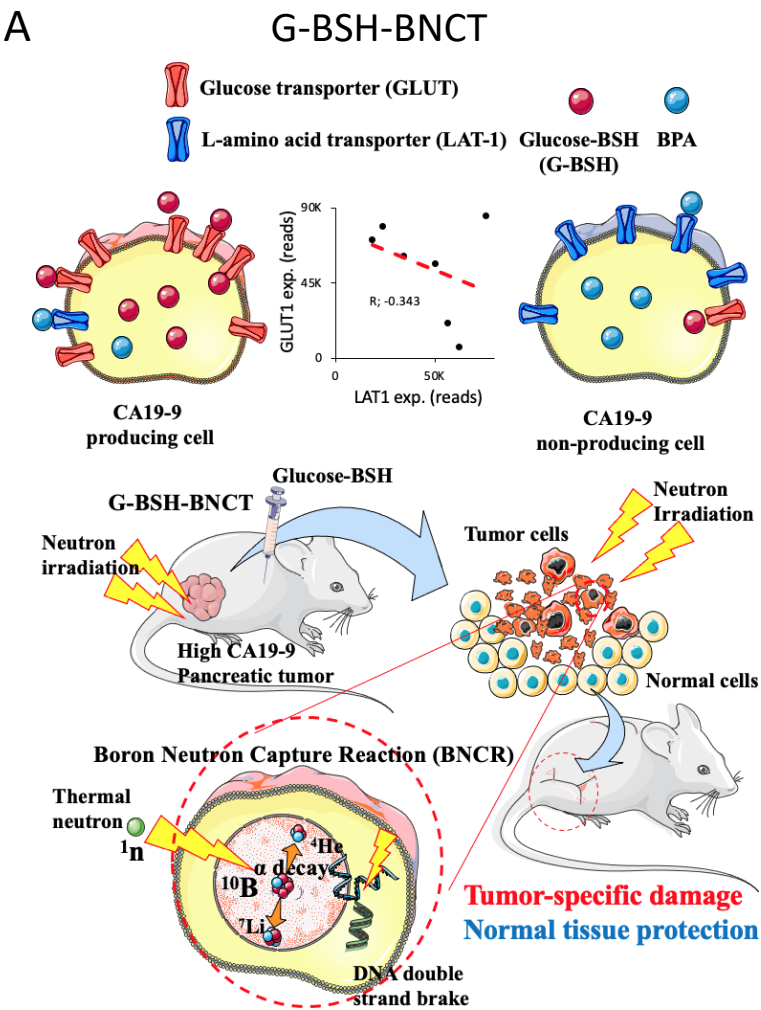
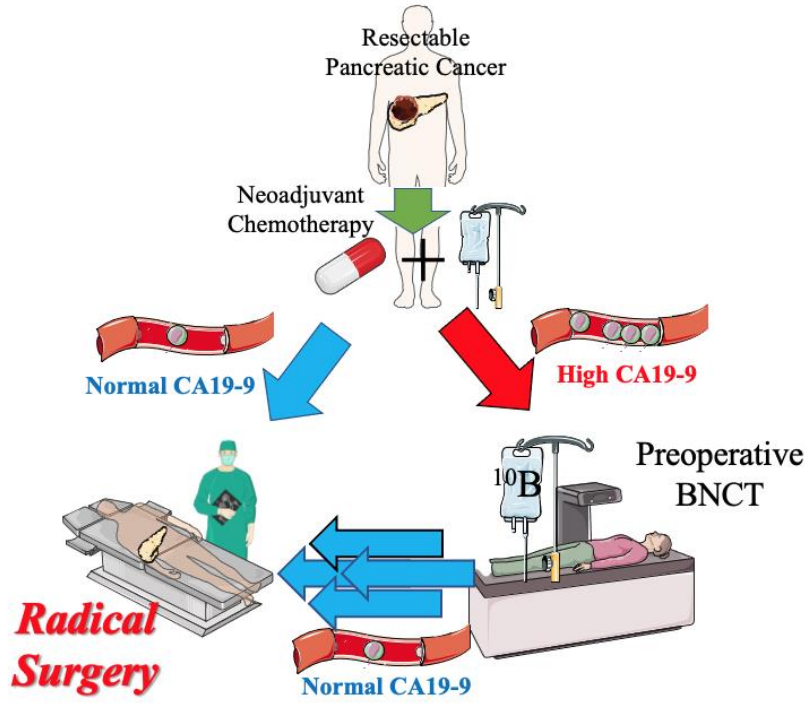


Fig. 8



B Future clinical application image of G-BSH-BNCT



Caption

Fig. 1 Synthesis of a new glucose-bound boron drug (Glucose-BSH).

(A) Synthesis of a new glucose-bound boron drug (Glucose-BSH). (B) ^1H NMR spectrum of Actylated Glucose-BSH in D_2O . (C) Fourier transform infrared spectroscopy (FT-IR) spectrum of Actylated Glucose-BSH. (D) Electrospray ionization mass spectrometry (ESI-MS) spectrum in positive mode of Actylated Glucose-BSH. (E) The magnified view of ESI-MS of Actylated Glucose-BSH.

Fig. 2 Glucose transporter expression analysis in CA19-9 producing and non-producing human pancreatic cancer cells.

(A,B) mRNA expression analysis on the basis of RNA-seq data of LAT1 and GLUT1 (A) and LAT1 and GLUT3 (B) in human pan-cancer cell lines (1076 lines) using Cancer Cell Line Encyclopedia (CCLE) (A: $R=-0.009$, B: $R=0.087$) (C,D) mRNA expression analysis of LAT1 and GLUT1 (C) and LAT1 and GLUT3 (D) in human pancreatic cancer cells (seven lines) using CCLE on the basis of RNA-seq data (C: $R=-0.343$, D: $R=0.245$) (E) Comparison of GLUT1 expression in CA19-9-secretory and -non-secretory pancreatic cancer cells ($P=0.051$) (F), Evaluation of CA19-9 secretion into the culture medium of human pancreatic cancer cells by ELISA (G) Evaluation of CA19-9 production in the cytoplasm by fluorescence immunostaining (red: CA19-9, blue: nuclei) (H) Analysis of GLUT1/3 and LAT1/2 protein expression in CA19-9 producing human pancreatic cancer cells and CA19-9 non-producing pancreatic cancer cells (I) Analysis of GLUT1/3 and LAT1/2 gene expression in CA19-9 producing and CA19-9 non-producing human pancreatic cancer cells using CCLE

Fig. 3 Evaluation of cellular uptake of Glucose-BSH, a novel boron drug.

(A) Chemical structure of Glucose-BSH, a new boron agent, and existing boron agents BSH and BPA. (B) Glucose-BSH, BSH, and BPA were administered at $100\text{ }\mu\text{M}$ in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells, and the intracellular boron concentration was measured 24 h later using ICP (red: Glucose-BSH, green: BSH, blue: BPA). (C) Glucose-BSH $100\text{ }\mu\text{M}$ was administered in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells (four lines) and intracellular localization and uptake were observed using fluorescent immunostaining at 2, 6, and 12 h after administration (red: BSH, green: phalloidin, blue: nuclei). (D,E) CA19-9 producing and non-producing human pancreatic cancer cells were treated with boron drugs at matched B10 concentrations (Glucose-BSH 50, $100\text{ }\mu\text{M}$,

BSH 50, 100 μ M, BPA600, 1200 μ M) and 24 h later, the intracellular boron concentration was measured using ICP (red: Glucose-BSH, green: BSH, blue: BPA).

Fig. 4 Evaluation of glucose-BSH cellular uptake in response to changes in glucose concentration and inhibition of uptake by glucose inhibitors (WZB117) in a simulated cancer microenvironment

(A) CA19-9 producing and non-producing human pancreatic cancer cells were incubated with varying glucose concentrations (0, 0.25, 0.5, 0.75, 1.0, 2.0 g/L) and cell viability was measured after 24 and 48 h. Cell viability was confirmed even at a glucose concentration of 0.25 g/L. (B) CA19-9 producing and non-producing human pancreatic cancer cells were incubated with varying glucose concentrations (0.5, 2, 4.5 g/L) and the intracellular boron concentration was measured using ICP after 24 h. (C) Glucose concentrations in the culture medium of CA19-9 producing and non-producing human pancreatic cancer cells were varied (0.5, 2, 4.5 g/L), and protein expression of GLUT1, GLUT3, and LAT1 was determined after 36 h. (D) CA19-9 producing human pancreatic cancer cells were cultured in different concentrations of WZB117 (0, 1, 5, 10 μ M) for 24 h and then incubated with 100 μ M of Glucose-BSH for 24 h. The intracellular boron concentration was measured using ICP.

Fig. 5 Cell-killing effect of BNCT with Glucose-BSH in human pancreatic cancer cells.

(A) Panc0403 cells (CA19-9 producing human pancreatic cancer cells) and PANC-1 cells (CA19-9 non-producing human pancreatic cancer cells) were treated with 100 μ M of each boron drug (BPA, BSH, Glucose-BSH) for 24 h and then irradiated with neutrons. After irradiation, cells were cultured for 14 days and subjected to colony formation assay. (B) The graphs show the irradiation doses in panc0403 and PANC-1 cells treated with boron drugs (BPA, BSH, and Glucose-BSH).

Fig. 6 Anti-tumor effect of BNCT with Glucose-BSH on a pancreatic cancer in vivo model.

(A) Panc0403 cells (CA19-9 producing human pancreatic cancer cells) were transplanted into the right-side abdomen of mice to create a pancreatic cancer subcutaneous tumor model. Glucose-BSH (75 mg/kg) was administered intraperitoneally. Uptake concentration in organs was measured using ICP. (B) The tumor to normal ratio (T/N ratio) (blue: skin, yellow: pancreas) and tumor to blood ratio (T/B) (red) were determined at various time points (0.5, 1, 2, 3, 6, 12 h) after intraperitoneal administration of Glucose-

BSH in the subcutaneous tumor model of pancreatic cancer. (C) A panc0403 pancreatic carcinoma subcutaneous tumor model was created and 75 mg/kg of BPA, BSH, or Glucose-BSH was administered intraperitoneally on day 7 after transplantation. Neutron irradiation was performed at KURRI 1 h after administration, and tumor volume was monitored. (D) Total biological dose (Gy-Eq) from neutron irradiation in the control and boron-drug-treated groups (n=6). (E) Representative subcutaneous tumors in the control and boron drug groups on day 35 after neutron irradiation are shown.

Fig. 7 Mouse orthotopic model of pancreatic cancer and results of preclinical studies of neutron irradiation of pancreatic cancer patients

(A) Photograph of an orthotopic model of pancreatic cancer using the Panc0403 cell line (B) HE-stained histopathology results of pancreatic cancer sites (scale bar 500µm) (C) Fluorescent immunostaining using HLA class I (GFP) and nuclear staining (blue) in pancreatic cancer area (scale bar 500µm) (D) ICP results of boron pharmacokinetics in tumor and various organs of mouse pancreatic cancer orthotopic model after Glucose-BSH intraperitoneally administration (75 mg/kg) (0.5, 1, 2, 4h). (E) The tumor to normal ratio (T/N ratio) and tumor to blood ratio (T/B) (red) in mouse pancreatic cancer orthotopic model (0.5, 1, 2, 4 h). (F, G, H) Images of irradiation of pancreatic cancer patients with Glucose-BSH BNCT (F: two external neutron irradiation of pancreatic head cancer, G: open irradiation of pancreatic head cancer, H: external neutron irradiation of lateral abdomen of pancreatic tail cancer). (I, J, K, L, M, N) Glucose-BSH BNCT dose volume histogram (DVH) and BNCT maximum and average doses (I, L: two external neutron irradiation of pancreatic head cancer, J, M: open irradiation of pancreatic head cancer, H, K: lateral abdominal external neutron irradiation of pancreatic tail cancer) Simulation with a minimum tumor dose of 20 Gy-Eq.

Fig. 8 Abstract of this experiment and image of clinical application of BNCT using Glucose-BSH

(A) CA19-9 producing pancreatic cancer cells express more GLUT and CA19-9 non-producing pancreatic cancer cells express less GLUT. A subcutaneous tumor model was created by transplanting CA19-9 producing pancreatic cancer cells into BALB-c/nu-nu mice and using Glucose-BSH BNCT showed potent anti-tumor effects. (B) Based on the results of this experiment, we believe that it will be possible to control CA19-9 levels by preoperative BNCT (external or open irradiation) for patients with localized pancreatic cancer who have high preoperative blood CA19-9 levels and are not

eligible for radical surgery, and to make more patients eligible for second-stage radical surgical resection.

Highlights

- Boron neutron capture therapy (BNCT) is a chemoradiotherapy using boron agents and neutron irradiation; the cancer accumulation capacity of boron agents is the key to its application in pancreatic cancer.
- We successfully developed a boron-based drug targeting a glucose transporter that is highly expressed in pancreatic cancer.
- BNCT using the sugar-binding boron drug developed in this study is a novel therapy as BNCT targeting pancreatic cancer with high CA19-9 levels.