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Tumor-specific delivery of BSH-3R for boron neutron capture therapy and positron emission tomography imaging in a mouse brain tumor model

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ABSTRACT

Glioblastoma, a malignant brain tumor with poor disease outcomes, is managed in modern medicine by multimodality therapy. Boron neutron capture therapy (BNCT) is an encouraging treatment under clinical investigation. In malignant cells, BNCT consists of two major factors: neutron radiation and boron uptake. To increase boron uptake in cells, we created a mercapto-closo-undecahydrododecaborate ($[B12HnSH]^{2-}2Na^{+}$, BSH) fused with a short arginine peptide (1R, 2R, 3R) and checked cellular uptake *in vitro* and *in vivo*. In a mouse brain tumor model, only BSH with at least three arginine domains could penetrate cell membranes of glioma cells *in vitro* and *in vivo*. Furthermore, to monitor the pharmacokinetic properties of these agents *in vivo*, we fused BSH and BSH-3R with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA); DOTA is a metal chelating agent for labeling positron emission tomography (PET) probe with ^{64}Cu. We administered BSH-DOTA-^{64}Cu and BSH-3R-DOTA-^{64}Cu to the tumor model through a mouse tail vein and determined the drugs' pharmacokinetics by PET imaging. BSH-3R showed a high uptake in the tumor area on PET imaging. We concluded that BSH-3R is the ideal boron compound for clinical use during BNCT and that in developing this compound for clinical use, the BSH-3R PET probe is essential for pharmacokinetic imaging.

1. Introduction

Glioblastoma multiforme (GBM) is the most common malignant central nervous system primary tumor that has been incurable for decades. It is difficult to remove all tumor cells without causing severe damage to the brain. Recently, chemotherapy using temozolomide against GBM has been found to prolong survival by 2.5 months [1]. Boron neutron capture therapy (BNCT) against GBM and various malignant cancers has recently drawn attention as a new and promising treatment option [2]. BNCT is a particle radiation therapy that provides a way to selectively destroy malignant cells and spare normal cells [3,4]. BNCT is based on nuclear capture and fission reactions of the ^{10}B atom with low energy thermal/epithermal neutrons to yield high linear energy transfer α particles and recoiling of ^{7}Li nuclei. Radiation damage is limited to cells containing ^{10}B because of the short trajectory of these particles (9–10 um; approximately one cell diameter). Thus, if ^{10}B agents can selectively target tumor cells, side effects typically associated with ionizing radiation can be avoided [5,6].

BNCT has been clinically applied for the treatment of malignant brain tumors, malignant melanoma, head and neck cancers, and hepatoma [7–10]. BNCT consists of neutron radiation toward the affected site and boron delivery to tumor cells. The development of a hospital neutron radiation generator has been going on smoothly; recently, upon the approval of the Japan Health, Labour and Welfare Ministry, a clinical trial on a new neutron generator device has already started [11]. However, we have encountered several problems with the boron compounds during clinical use of BNCT. Many

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kinds of boron compounds, including amino acids, nucleic acids, and liposomes, have been reported as boron delivery agents (boron carriers) during BNCT, but only two compounds, p-borono-L-phenylalanine (BPA) and disodium mercaptoundecahydrododecaborate ([B12HnSH]$^{2-}$2Na^{+}, BSH), are clinically used in treatment of cancer with BNCT. BPA, an essential amino acid analog, is actively carried to brain tumors, and it can be detected on positron emission tomography (PET) imaging by ^{18}F-BPA [12]. However, it also accumulates in normal brain tissue and does not accumulate in slowly proliferating malignant cells. In contrast, BSH contains abundant ^{10}B and accumulates with enhanced permeability and retention (EPR) in a tumor region but less in normal tissue. However, BSH is present only in intercellular spaces and does not get into the cells. Therefore, the therapeutic effect with BSH during BNCT for GBM is insufficient [2].

To overcome this limitation of BSH, several drug delivery systems using therapeutic doses of BSH-containing pharmacophores have been reported [4,5]. Protein transduction therapy with cell-membrane penetrating peptides and a protein/peptide transduction domain has marked advantages in moving several molecules (e.g., protein, peptide, small-interfering RNA) into cells without toxicity, *in vitro* and *in vivo* [13–17]. In a previous report, we successfully transduced BSH into cells using a polyarginine peptide (11R) [18]. However, this BSH peptide has several limitations, such as difficulty in synthesizing BSH-11R and the lack of a pharmacokinetic imaging system, that have hindered its applications in clinical use [18].

In this study, we attempted to develop a BSH peptide-type boron formulation that can be readily synthesized and that will enable easy PET imaging. We aim to introduce a new breakthrough on the BSH peptide that may be clinically used as a second generation boron compound during BNCT.

2. Materials and methods

2.1. Synthesis of BSH-arginine conjugates containing Tmr or DOTA

Peptides consisting of BSH, arginine (Arg) and 5 (and 6)-tetramethylrhodamine (Tmr) [BSH-nR-Tmr (n = 1, 2, and 3)] or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, as adduct to epsilon amino group of Lys) or BSH-nR-DOTA (n = 0, 2, and 3) were synthesized by conventional Fmoc-based solid-phase peptide synthesis (SPPS)1 before a liquid-phase synthesis for conjugation of a peptide with BSH. Tmr-Lys-Arg-Arg-Arg-NH2, Tmr-Lys-Arg-Arg-NH2, and Tmr-Lys-Arg-NH2 were prepared by SPPS and then connected by a maleimide crosslinker (N-succinimidyl 4-maleimidobutyrate) to an amino group on Lys of the peptide in dimethyl sulfoxide. The obtained compounds were linked to BSH via a thioether linkage between the maleimide group of the compound and the sulfhydryl group of BSH in an aqueous solution (100 mM PBS, pH 7.2).

Next, H-Arg-Arg-Arg-Lys(DOTA)-NH2, H-Arg-Lys(DOTA)-NH2, and H-Lys(-DOTA)-NH2, containing the maleimide crosslinker in the N-terminus, were prepared by SPPS. Finally, BSH-nR-Tmr and BSH-nR-DOTA were purified by high-performance liquid chromatography (HPLC; C18 column) using acetonitrile-water containing 0.1% trifluoroacetic acid (TFA) as an eluent, and they were further characterized by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (Fig. S1a, b).

2.2. Glioma cell lines

U87ΔEGFR glioma cell lines, which stably express constitutively active EGFRvIII, were used in all experiments. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako) with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 [19].

2.3. Cell proliferation assay (WST-1 assay)

2.3.1. Cell viability assay

After the glioma cells (5 × 10^3/well) were seeded in 96-well flat-bottomed plates, they were cultured in DMEM containing 10% FBS for 24 h. The cells were then supplemented with varying concentrations of either BSH or BSH peptide and were further incubated for 24 h. Cell viability was measured everyday using the WST-1 assay according to the manufacturer's instructions (Roche Applied Science) [20].

2.4. Immunohistochemical analysis (IHC) and measurement of ^{10}B concentration in vitro

IHC was performed to analyze the distribution of BSH *in vitro*. The cells were incubated for 24 h with the BSH or BSH peptides. After incubation of boron compounds, the cells were thoroughly washed with PBS twice, fixed with 4% paraformaldehyde (PFA) for 15 min, and incubated with anti-BSH mouse mAb [18]. The secondary antibody was Alexa-Fluor 488, a conjugated mouse IgG. Fluorescence signals were observed using a confocal laser microscope (FluoView; Olympus, Japan).

To detect ^{10}B in cells, the BSH or BSH peptides were added to 6 cm dishes. After 1 h, 2 h, 4 h, 12 h, and 24 h of incubation, the cells were washed with PBS, dissolved in 300 μL HNO_3 (ultra-trace analysis grade, Wako) for 30 min, and diluted with 2.7 mL water. After passing through a filter (F2513-16, Thermo, USA), the ^{10}B concentration was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES; Vista Pro, Seiko Instruments, Japan) as previously described [21].

2.5. Brain tumor model and detection of BSH-peptide in vivo

The U87ΔEGFR cells (3 × 10^5 cells/3 μL) were injected into the corpus striatum of 7–9 week-old female nude mice (15–20 g, BALB/c Slc-nu/nu, Japan SLC) as previously described [18]. After two weeks, 200 nmol of boron per 200 μL of BSH or BSH peptide was intravenously administered via the tail of the tumor-bearing mice. After 24 h, the mice were sacrificed, and the brains were placed in PFA. Brain sections of 10 um thickness were cut on a microtome (CM 1850; Leica Microsystems, Wetzlar, Germany). IHC was conducted and observed with confocal laser microscopy (Olympus) [18].

To analyze the distribution of ^{10}B, 200 nmol of boron per 200 μL of BSH or BSH peptide was administered via the tail. After 6 h, 12 h, and 24 h, the tumor, normal brain, blood, liver, and kidney were excised and weighed. These tissues were digested with 2 mL HNO_3 at 90 °C for 1.5 h and were diluted with 3 mL water. After passing through a filter, the ^{10}B concentration was measured by ICP-AES.

2.6. ^{64}Cu labeling

Copper-64 was produced by a cyclotron (HM-12 cyclotron; Sumitomo Heavy Industries, Ltd., Tokyo, Japan) and was purified according to a previously reported method [22]. The ^{64}Cu-DOTA-peptides were prepared by incubating 2 nmol of each conjugate with approximately 370 MBq of ^{64}CuCh in a phosphate buffer (0.1 mol/L, pH 5.5) at 40 °C for 30 min. The reaction solution was purified with a JASCO PU-2080 HPLC system (JASCO Co., Tokyo, Japan), equipped with a YMC-Pack ODS-AM reverse-phase column (10 × 250 mm; YMC Co., Ltd., Tokyo, Japan), using isocratic 20% acetonitrile/0.1% TFA in water. The radioactive peak containing ^{64}Cu-DOTA-peptide was collected, the solvent was evaporated, and the activity was reconstituted in phosphate-buffered saline (PBS). The radiochemical purity of the radiolabeled preparations was analyzed by RP-HPLC on a Shimadzu LC-10Ai system (Shimadzu Co., Japan). A YMC-Pack ODS-AM reverse-phase column (4.6 × 150 mm; YMC Co., Ltd.) was used at a flow rate of 1 mL/min with the following buffer systems: buffer A, 0.1% TFA in water; buffer B, acetonitrile; and a gradient of 95% buffer A at 0–5 min and 95% buffer A to 60% buffer A at 5–25 min. Absorbance was detected at 214 nm using a Shimadzu SPD-20A detector; radioactivity was monitored using an in-line Raytest Gabi Star NaI(Tl) detector (Agilent Technologies, California, USA).

BSH-0R-DOTA and BSH-3R-DOTA were radiolabeled with ^{64}Cu by the addition of ^{64}CuCl2 in 0.1 M phosphate buffer (pH 5.5), followed by a 30 min incubation period at 40 °C. The radiolabeled complex was purified by radio-HPLC. The radioactive peak containing the desired product was collected and rotary-evaporated to remove the solvent. The purified products were reconstituted in phosphate buffer to 50 nmol/100 μL.

2.7. PET and CT imaging, and biodistribution study

^{64}Cu-DOTA-peptides (3.57 ± 1.35 MBq) were injected into the tumor-bearing mice via the tail vein. At 3 h, 6 h, 12 h, and 24 h after injection, the mice were anesthetized with 1.5% isoflurane and were imaged on a small-animal PET imaging system with a spatial resolution of 1.4 mm (Clarivivo-PET; Shimadzu Co.). During the acquisition period, a thermostat-controlled heater maintained the body temperature of the mice. After PET acquisition, the mice were moved to a CT scanner (Aquilion; Toshiba Medical Systems Co., Tokyo, Japan) to scan for anatomy. PET images were reconstructed with two iterations of 3-dimensional dynamic row-action maximum likelihood algorithm. Quantitative analysis of ^{64}Cu-DOTA-peptide uptake was performed using PMOD software (version 3.3; PMOD Technologies Ltd. Zurich, Switzerland). The PET and CT image sections of the same location were manually fused and volumes of interest (VOIs) were defined on the tumor, normal brain, heart, liver, and thigh muscle, guided by the CT image. Tissue activity was generated from VOIs and converted to percentage of the injected dose per cubic centimeter of tissue %ID/cc. In addition, biodistribution studies were conducted at 6 h and 24 h after injection of a similar dose of ^{64}Cu-DOTA-peptides. The tumors and organs were excised, rinsed for residual blood, weighed, and counted for radioactivity in a calibrated well-type gamma counter (AccuFLEX gamma 7000; Hitachi Aloka Medical, Ltd., Tokyo, Japan). Tissue activity was expressed as percentage of the injected dose per gram of tissue (%ID/g).