I-1. PROJECT RESEARCHES

Project 8

PR8 Analyzing Tumor Microenvironment and Exploiting its Characteristics in Search of Optimizing Cancer Therapy Including Neutron Capture Therapy

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BACKGROUNDS AND PURPOSES: Human solid tumors contain moderately large fractions of quiescent (Q) tumor cells that are out of the cell cycle and stop cell division, but are viable compared with established experimental animal tumor cell lines. The presence of Q cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, which is another consequence of poor vascular supply. As a result, Q cells are viable and clonogenic, but stop cell division. In general, radiation and many DNA-damaging chemotherapeutic agents kill proliferating (P) tumor cells more efficiently than Q tumor cells, resulting in many clonogenic Q cells remaining following radiotherapy or chemotherapy. Therefore, it is harder to control Q tumor cells than to control P tumor cells, and many post-radiotherapy recurrent tumors result partly from the regrowth of Q tumor cells that could not be killed by radiotherapy. Similarly, sufficient doses of drugs cannot be distributed into Q tumor cells mainly due to heterogeneous and poor vascularity within solid tumors. Thus, one of the major causes of post-chemotherapy recurrent tumors is an insufficient dose distribution into the Q cell fractions.

With regard to boron neutron capture therapy (BNCT), with ^{10}B-compounds, boronophenylalanine-^{10}B (BPA) increased the sensitivity of the total cells to a greater extent than mercaptoundecahydrododecaborate- ^{10}B (BSH). However, the sensitivity of Q cells treated with BPA was lower than that in BSH-treated Q cells. The difference in the sensitivity between the total and Q cells was greater with ^{10}B-compounds, especially with BPA. These findings concerning the difference in sensitivity, including other recovery and reoxygenation following neutron irradiation after ^{10}B-compound administration were mainly based on the fact that it is difficult to deliver a therapeutic amount of ^{10}B from ^{10}B-carriers throughout the target tumors, especially into intratumor hypoxic cells with low uptake capacities.

Hypoxia is suggested to enhance metastasis by increasing genetic instability. Acute, but not chronic, hypoxia was reported to increase the number of macroscopic metastases in mouse lungs. We recently reported the significance of the injection of an acute hypoxia-releasing agent, nicotinamide, into tumor- bearing mice as a combined treatment with γ-ray irradiation in terms of repressing lung metastasis. As the delivered total dose increased with irradiation, the number of macroscopic lung metastases decreased reflecting the decrease in the number of clonogenically viable tumor cells in the primary tumor. The metastasis-repressing effect achieved through a reduction in the number of clonogenic tumor cells by irradiation is much greater than that achieved by releasing tumor cells from acute hypoxia. On the other hand, more ^{10}B from BPA than from BSH could be distributed into the acute hypoxia-rich total tumor cell population, resulting in a greater decrease in the number of highly clonogenic P tumor cells with BPA-BNCT than with BSH-BNCT and with neutron beam irradiation only. BPA-BNCT rather than BSH-BNCT has some potential to decrease the number of lung metastases, and an acute hypoxia- releasing treatment such as the administration of nicotinamide, bevacizumab, wortmannin or thalidomide may be promising for reducing numbers of lung metastases. Consequently, BPA-BNCT in combination with the treatment using these agents may show a little more potential to reduce the number of metastases. Now, it has been elucidated that control of the chronic hypoxia-rich Q cell population in the primary solid tumor has the potential to impact the control of local tumors as a whole, and that control of the acute hypoxia-rich total tumor cell population in the primary solid tumor has the potential to impact the control of lung metastases.

The aim of this research project is focused on clarifying and analyzing the characteristics of intratumor microenvironment including hypoxia within malignant solid tumors and optimizing cancer therapeutic modalities, especially radiotherapy including BNCT in the use of newly-developed ^{10}B-carriers based on the revealed findings on intratumor microenvironmental characteristics.

RESEARCH SUBJECTS:

The collaborators and allotted research subjects (ARS) were organized as follows;

ARS-1 (30P8-1): Optimization of Radiation Therapy Including BNCT in terms of the Effect on a Specific Cell Fraction within a Solid Tumor and the Suppressing Effect of Distant Metastasis. (S. Masunaga,*et al.*)

ARS-2 (30P8-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers. (H. Nagasawa, *et al.*)

ARS-3 (30P8-3)*: Search and Functional Analysis of Novel Genes that Activate HIF-1, and Development into Local Tumor Control. (H. Harada, *et al.*)

ARS-4 (30P8-4)*: Radiochemical Analysis of Cell Lethality Mechanism in Neutron Capture Reaction. (R. Hirayama, *et al.*)

ARS-5 (30P8-5): Development of Neutron Capture Therapy Using Cell-Membrane Fluidity Recognition Type Novel Boron Hybrid Liposome. (S. Kasaoka, *et al.*)

ARS-6 (30P8-6): Drug Delivery System Aimed at Adaptation to Neutron Capture Therapy for Melanoma. (T. Nagasaki, *et al.*)

ARS-7 (30P8-7): Molecular Design, Synthesis and Functional Evaluation of Hypoxic Cytotoxin Including Boron. (Y. Uto, *et al.*)

ARS-8 (30P8-8): Bystander Effect on Malignant Trait of Tumor Cells by Irradiation. (H. Yasui, *et al.*)

ARS-9 (30P8-9): Analysis of the Response of Malignant Tumor to BNCT. (M. Masutani, *et al.*)

ARS-10 (30P8-10): Cell Survival Test by Neutron Capture Reaction Using Boron Compound and Inhibitory Effect on Tumor Growth. (K. Nakai, *et al.*)

ARS-11 (30P8-11): Multilateral Approach Toward Realization of Next Generation Boron Neutron Capture Therapy. (Y. Matsumoto, *et al.*)

ARS-12 (30P8-12): Analysis of Radiosensitization Effect through Targeting Intratumoral Environmental. (Y. Sanada, *et al.*)

ARS-13 (30P8-13)*: Exploratory Research on the Optimal Administration of ^{10}B Compound Aiming at New Enforcement Method of Neutron Capture Therapy (S. Masunaga *et al.*)

ARS-14(30P8-14) *: Examination of Cancer Cell Accumulation Property of New Boron Agent (CbaP14). (J. Hiratsuka, *et al.*)

(*There was no allocated time for experiments using reactor facilities during their operation periods of FY 2018.**)**

PR8-1

Estimation of Therapeutic Efficacy of BCNT Based on the Intra- and Intercellular Heterogeneity in ^{10}B-Distribution

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INTRODUCTION: Two types of dose were proposed for use in the treatment planning of boron neutron capture therapy (BNCT) for expressing its high relative biological effectiveness (RBE). On one hand, the RBE -weighted dose is the sum of the absorbed doses weighted by fixed RBE for four dose components of BNCT, which are attributable to ^{10}B(n,α)^{7}Li, ^{14}N(n,p)^{14}C, and ^{1}H(n,n)p reactions as well as photons, respectively. On the other hand, photon-isoeffective dose is the photon dose to give the same biological effect calculated considering the dose dependence of RBE and the synergetic effect between different types of radiation [1]. In this study, the depth distributions of the two types of dose in a phantom placed at an accelerator-based BNCT field were calculated using Particle and Heavy Ion Transport code System, PHITS [2], coupled with an extended stochastic microdosimetric kinetic (SMK) model [3].

MATERIALS AND METHODS: The basic flow of the calculation procedures is shown in Fig. 1. In the PHITS simulation, a cylindrical phantom with the radius of 10 cm and the depth of 20 cm composed of ICRU soft tissue was placed at the beam port of the Linac-based BNCT facility in University of Tsukuba [4]. The absorbed doses were determined from the calculated neutron and photon fluxes multiplied with their corresponding kerma factors for each dose component. The calculated absorbed doses were converted to RBE-weighted dose and photon-isoeffective dose, using the extended SMK model. The parameters used in the model were evaluated by the least-square fitting of the surviving fractions of SCC VII murine squamous cell carcinomas obtained from *in vivo/in vitro* experiments of tumor-bearing mice treated by BNCT [5].

RESULTS AND DISCUSSION: Fig. 2 shows the RBE-weighted dose and photon-isoeffective dose in the phantom administrated with BPA or BSH, or without ^{10}B. The absorbed doses at the entrance of the phantom without ^{10}B were normalized to 2 and 10 Gy in panels (A) and (B), respectively. It is evident from Fig. 2 that the photon-isoeffective doses are larger than the corresponding RBE-weighted doses in panel (A), while the reverse is true in panel (B) except at the locations deeper than 7 g/cm^2. The depth dependence was more apparent in the RBE-weighted doses, which decreased more dramatically with increasing depth. Such tendency is mainly due to the consideration of the dose dependence of RBE in the calculation of the photon-isoeffective dose because RBE decreases with an increase in the absorbed dose. The consideration of the synergetic effect also causes the difference, which raises the photon-isoeffective dose by approximately 10-20%. More detailed discussions can be found in our recently published paper [6].

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[1] S.J. Gonzalez, *et al.*, Phys Med Biol **62** (2017) 7938-7958.
[2] T. Sato *et al.*, J Nucl Sci Technol **55**, (2018) 684-690.
[3] T. Sato *et al.*, Sci Rep **8** (2018) 988.
[4] A. Masuda *et al.*, Appl Radiat Isot **127** (2017), 47-51.
[5] S. Masunaga *et al.*, Springerplus **3** (2014), 128.
[6] T. Sato *et al.*, Radiat Prot Dosim (2018) DOI: 10.1093/rpd/ncy235

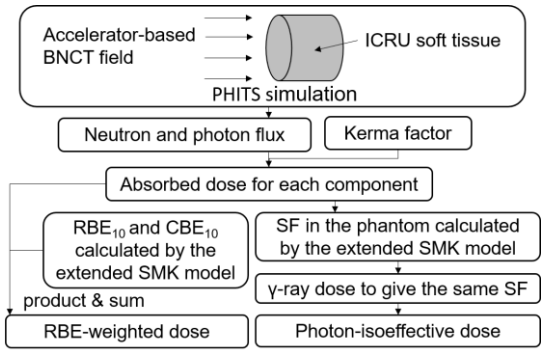

Fig. 1. Basic flow of the calculation carried out in this study.

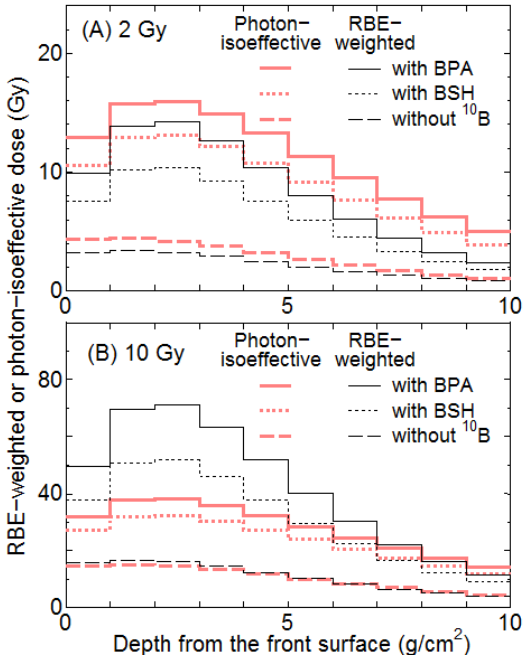

Fig. 2. Calculated RBE-weighted dose and photon -isoeffective dose in the phantom administrated with BPA or BSH, or without ^{10}B.

PR8-2 Design, synthesis and biological evaluation of lipopeptide conjugates of BSH for BNCT

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INTRODUCTION: Tumor selective delivery of sufficient quantity of ^{10}B is essential for the success of boron neutron capture therapy (BNCT). The clinically used boron carrier, sodium mercaptoundecahydro-closo-dodecaborate (BSH: Na2B12H11SH) does not penetrate the cell membrane directly, due to its highly hydrophilic and anionic properties. Here, we developed a new membrane-permeable boron cluster using the cell-penetrating lipopeptide pepducin as the vehicle for intracellular delivery of boron clusters. Pepducins are membrane-permeable, lipidated peptides, developed as allosteric modulators of the G-protein-coupled receptor (GPCR). We have previously developed fluorescence resonance energy transfer (FRET)-based pepducin probes to demonstrate the direct membrane penetration of a pepducin by fluorescence imaging in living cells. So, we designed and synthesized pepducin-BSH conjugates and performed structural optimization to improve cellular uptake. (Fig.1)

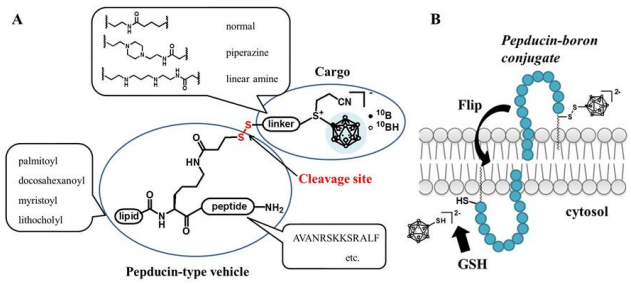
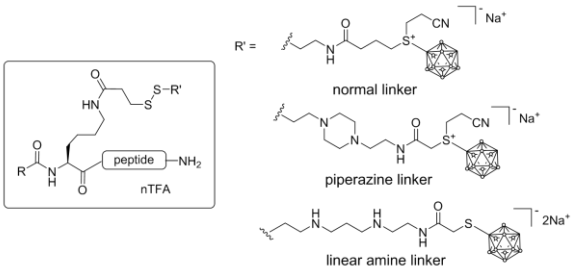


Fig. 1. Molecular design (A) and putative mechanism of intracellular delivery of pepdicin-BSH conjugates.

EXPERIMENTS: All compounds were synthesized based on sol-id-phase synthesis. (Table 1) T98G, cells were treated with the boron carriers (10 or 20 μM) at 37 °C for various times, then, washed with PBS three times, and dissolved in 200 μL HNO3 for 1 h. The boron concentrations of these extracts were measured by ICP-AES. To evaluate neutron sensitizing ability of the compounds, T98G cells were treated with 20 μM boron carriers for 24 h. Then the cells were washed with PBS, suspended in serum containing medium and aliquoted into Teflon tubes for irradiation. Cells were irradiated using the neutron beam at the Heavy Water Facility of the Kyoto University Research Reactor (KUR) operated at 1 MW power output. The survival rates of the irradiated cells were determined using conventional colony assays.

Table 1. Structures of new boron carriers.



Boron carrier	R	Sequence of peptide	linker
1a[a]	$C_{15}H_{31}$	**AVANRSKKSRALF**	
1b	$C_{15}H_{31}$	**KKSRALF**	
1c	$C_{15}H_{31}$	**NRSKKSRALF**	
1d	$C_{15}H_{31}$	**AVANRSK**	
1e	$C_{15}H_{31}$	**AVANRSKKSR**	
1f	$C_{15}H_{31}$	**ALFAVANRSKKSR**	
1g	$C_{15}H_{31}$	**ARVSAKNKASLRF**	
1h	$C_{15}H_{31}$	**FLARSKKSRNAVA**	
1i	$C_{15}H_{31}$	**AVANRPGKSRALF**	normal
1j	$C_{15}H_{31}$	**AVANRPKKSRALF**	
2a[a, b]	$C_{15}H_{31}$		
3a	CH_3		
3b	$C_{22}H_{19}$		
3c	$C_{13}H_{27}$		
3d	$C_{23}H_{38}OH$	**AVANRSKKSRALF**	
3e	$C_{23}H_{38}OCOCF_3$		
4[a]	$C_{15}H_{31}$		piperazine
5[a,b]	$C_{15}H_{31}$		linear amine

[a] Lipopeptide moiety is derived from P1pal-13. [b] No cyanoethyl group.

RESULTS: Among the pepducin-BSH conjugates, the most effectively incorporated and accumulated in the cells was compound **1a,** which has a peptide of 13 residues derived from the intracellular third loop of PAR1 and a palmitoyl group. Compound **1a** and compound **4** both showed a stronger radio-sensitizing effect than BSH on T98G cells under mixed-neutron beam irradiation. (Table 2)

Table 2. Plating efficiencies without irradiation, D_{10} values and the enhancing effects of boron carriers.

	PE without irradiation (%)	D_{10} value (Gy)	The enhancing effect
Control	19.0±4.4	4.17	-
BSH	32.9±3.6	4.32	0.97
1a	51.2±5.6	0.55	7.69
4	20.0±4.5	0.72	5.79

The results demonstrate that lipopeptide conjugation is apparently effective for enhancing intracellular delivery and accumulation of BSH and improving the cytotoxic effect of BNCT [1].

REFERENCES:

[1] A. Isono *et al.*, ChemMedChem., **14** (2019) 823-832.

PR8-3 Molecular mechanism underlying radioresistance of hypoxic tumor cells

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INTRODUCTION: Cancer cells are 2-3 times more radioresistant under hypoxic than normoxic conditions, which causes poor prognosis of patients with more hypoxic regions in their tumor tissues [1]. Although the radioresistance is known to be induced by biological mechanisms, at least in part, in a hypoxia-inducible factor 1 (HIF-1)-dependent manner, further studies are needed for better understanding of the nature of hypoxic cell radioresistance [2, 3].

EXPERIMENTS & RESULTS:
Here we found that hypoxia-inducible secretory protein 2, HISP2, which we recently identified as a novel hypoxia-responsive gene through our own DNA microarray analysis, causes radioresistance of hypoxic cancer cells in a HIF-1-independent manner (*in preparation*). qRT-PCR experiments confirmed that HISP2 mRNA levels were significantly upregulated under hypoxic conditions. ELISA experiments for culture media showed that the amount of secreted HISP2 protein levels increases under hypoxic conditions. The hypoxia-dependent increase in the HISP2 mRNA levels was abrogated by a transcription inhibitor, actinomycin D, suggesting that HISP2 were upregulated under hypoxia at transcription initiation levels. A loss-of-function study found that the hypoxia-dependent increase in the HISP-2 mRNA levels was significantly abrogated by silencing of HIF-1β, but not by that of HIF-1α and HIF-2α, suggested that the hypoxia-mediated expression is dependent on HIF-1β. A reporter gene assay with the use of an expression vector for EGFP-53BP1M fusion protein demonstrated that HISP2-overexpression decreased the number of DNA double strand breaks after irradiation. Clonogenic cell survival assay showed that cancer cells acquired radioresistance when transfected with HISP2-overexpression vector. All of these data suggested that HISP2 proteins are secreted under hypoxic conditions in a HIF-1-independent but HIF-1β-dependent manner and decrease the number of double-strand break after irradiation, leading to radioresistance of cancer cells in an autocrine fashion.

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[3] Nagao A, Kobayashi M, Koyasu S, Chow CCT, *Harada H. HIF-1-dependent reprogramming of glucose metabolic pathway of cancer cellsand its therapeutic significance. *Int J Mol Sci*. 20:238. 2019.

PR8-4 Membrane-Targeted Boron Neutron Capture Therapy with Boron Liposomes Composed of Dimyristoylphosphatidylcholine

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INTRODUCTION: There are many reports that membranes in cancer cells are relatively more fluid compared to healthy cells. Higher membrane fluidity in cancer cells closely relates to their invasive potential, proliferation, and metastatic ability [1]. Liposomes composed of dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenedodecylether were found to inhibit the growth of human promyelocytic leukemia cells without using any drugs [2]. In this study, we have developed a novel boron delivery system using the membrane-fluidity sensitive boron liposomes (MFSBLs) composed of DMPC and borocaptate (BSH)-conjugated chemical compounds for boron neutron capture therapy.

EXPERIMENTS: Octadecylamine and 1,2-dimyristoyl-sn-glycero-3-phosphorylethanolamine were conjugated with BSH using the optimal hetero-crosslinking agents for boron compounds. MFSBLs composed of DMPC, polyoxyethylenedodecylether and boron compounds at mole ratios of 8:0.9:1.1 were prepared by sonication method in 5% glucose solution at 45°C. The diameter of MFSBLs was measured with a light scattering spectrometer. The boron concentration was measured by inductively coupled plasma atomic emission spectrometry. B16F10 murine melanoma cells, COLO679 human melanoma cells and human fibroblast cells were incubated with 2-6 ppm of ^{10}B at 37℃ for 14 hours before neutron irradiation. The cells were rinsed twice in PBS and suspended in fresh medium. After neutron irradiation the cells were plated into plastic Petri dishes 60 mm in diameter at 200 cells per dish. They were incubated for an additional 7 days to allow colony formation.

RESULTS: MFSBLs had a mean diameter of 59.6 nm. High encapsulation efficiency value from 55% to 89% of ^{10}B in MFSBLs were obtained. MFSBLs had high stability (95-99%) in the retention of ^{10}B during storage at 4℃ for 4 weeks. All borocaptate-loaded formulations had low cytotoxic effects in human fibroblast cells. MFSBLs were efficiently fused to melanoma cells, but were inefficiently fused to human fibroblast cells. Thus, it is essential to elevate the ^{10}B concentration in melanoma cells, while maintain low levels of ^{10}B in normal fibroblast cells. The tumor/normal ratio (T/N ratio) was 2.1-3.0. As shown in Fig. 1, MFSBLs showed higher suppression of growth of murine and human melanoma cells than BSH solution. This result suggested novel MFSBLs is useful for ^{10}B carrier on BNCT for melanoma.

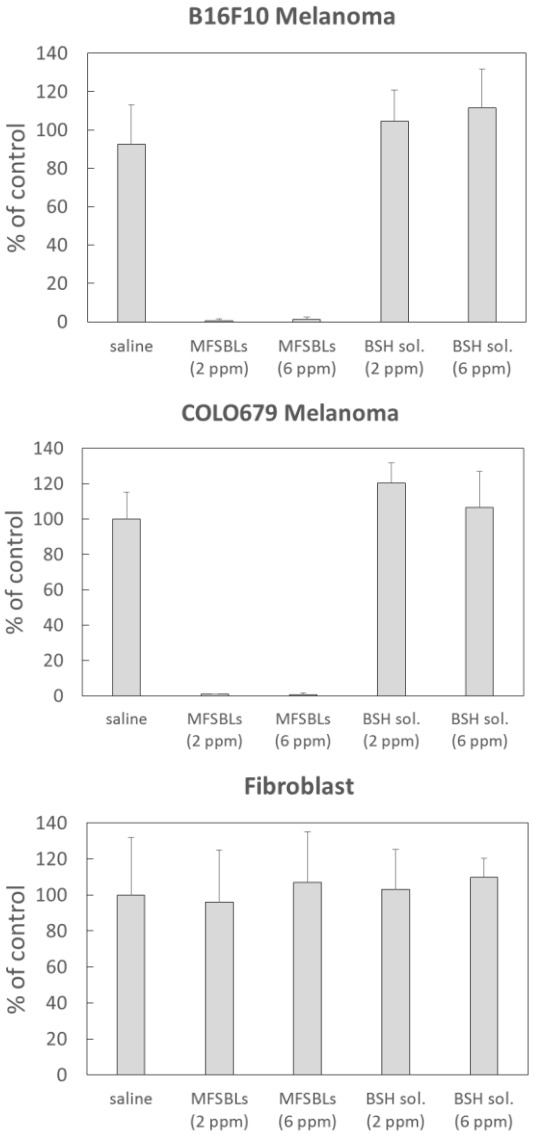

Fig. 1. Suppression of the colony formation of B16F10 cells, COLO679 melanoma cells and fibroblast cells after in vitro BNCT.

REFERENCES:

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[2] Y. Matsumoto *et al.* Int. J. Cancer, **115** (2005).

PR8-5 GLUT1-mediated endocytosis could be a major pathway for internalisation of kojic acid-appended carborane conjugate into melanoma cells

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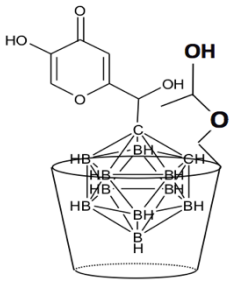
INTRODUCTION: Receptor-mediated endocytosis is potent pathways for the internalization of drugs into target cells. It is well known that the expression level of glucose transporter and uptake of glucose are increased in cancer cells [1]. We have previously reported the inclusion complex of kojic acid-appended carborane (CKA) with hydroxypropyl-β-cyclodextrin (HP-β-CD) as a novel boron agent is potent for melanoma targeting BNCT. CKA/HP-β-CD complex showed melanoma cells selectivity, unique nuclear localization, and high tumor-suppression effect on BNCT toward melanoma-bearing mice. Herein, mechanism of high efficient internalization of CKA/HP-β-CD complex into melanoma cells is evaluated.



Structure of CKA/HP-β-CD

EXPERIMENTS: B16BL6 cells were maintained in RPMI containing 10% FBS and 1% penicillin/streptomycin. B16BL6 cells seeded on the 6 well plate at the concentration of 2.0×10^5 cells/well. The cells were exposed to CKA/HP-β-CD complex (10 ppm) and the cells were corrected at each time point (12 and 24 hr). The involvement of GLUT1 on the uptake of CKA is confirmed with not only a small-molecule inhibitor of GLUT1 [2] ,but also RNA interference-mediated reduction in GLUT1 [3]. The isolated cells were digested by using aqua regia with heating (95 ºC, 30 min; 115 ºC, 60 min). After centrifugation, the cellular uptake amounts were quantified by ICP-AES.

RESULTS AND DISCUSSION: As shown in Fig. 1 and 2, clear suppression of CKA uptake by melanoma cells were observed in the presence of WZB117 inhibitor and siRNA for GLUT1. Furthermore, CKA uptake obviously reduced when melanoma cells were incubated in the normal medium with glucose. These results suggested that the internalization of CKA/HP-β-CD complex into melanoma cells is enhanced with GLUT1-mediated endocytosis.

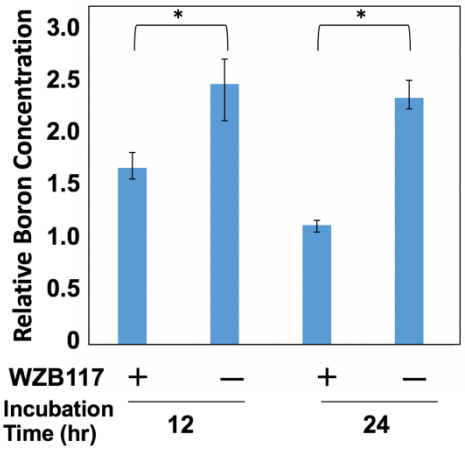


Fig.1 Suppression of CKA uptake by the inhibition of GLUT1 by WZB117. B16BL6 cells were incubated in the presence or absence of WZB117 (30 μM) for 12 and 24 hr. * denotes $P < 0.02$ between indicated treatments.

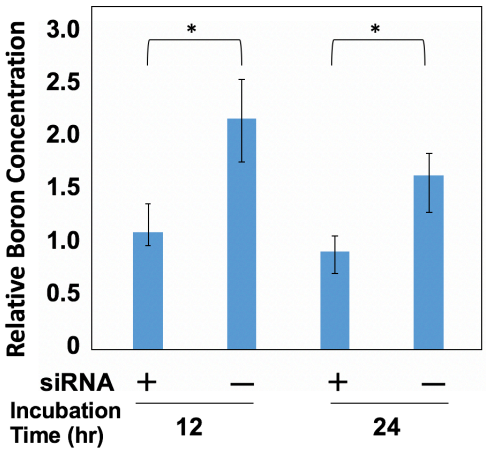


Fig.2 Suppression of CKA uptake by RNA interference-mediated reduction in GLUT1. B16BL6 cells were incubated in the presence or absence of siRNA (100 nM) for 12 and 24 hr. * denotes $P < 0.02$ between indicated treatments.

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PR8-6 Development of novel BPA-Tirapazamine hybrid BNCT agent targeting hypoxic tumor cells

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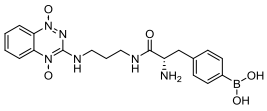
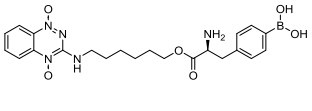
INTRODUCTION: Hypoxia is a ubiquitous environment of cancer, it has a resistance to anticancer drugs and X-ray, and accelerated infiltration / metastasis is a problem. Boron neutron capture therapy (BNCT), a kind of radiotherapy, is a treatment method utilizing neutron capture reaction in tumor cells by accumulating ^{10}B in tumor tissue. Hybrid molecules of Tirapazamine (TPZ), as a hypoxic cytotoxin and the neutron scavenger *p*-borono-*L*-phenylalanine (BPA) are useful as selective BNCT agent for hypoxic tumors. Since therapeutic efficiency of BNCT depends on ^{10}B concentration, it is expected to provide an effective therapeutic effect for hypoxic cancer.

In this study, UTX-117 with amide linkage of TPZ and BPA and UTX-118 with ester linkage of TPZ and BPA were designed and synthesized, and antitumor activity and neutron sensitizing activity were evaluated. UTX-117 and UTX-118 exhibit hypoxia selective cytotoxicity and UTX-118 has improved uptake rate compared to BPA fructose complex. Further, UTX-117 and UTX-118 shows neutron sensitizing activity.

EXPERIMENTS: Tirapazamine derivatives and L-BPA were condensed to synthesize UTX-117 and UTX-118 (Fig.1). Using melanoma cells B16-F10 cells, the IC_{50} values of UTX-117 and UTX-118 were evaluated by WST-1 assay. The cell uptake of UTX-117 and UTX-118 was measured by ICP-AES, and the intracellular boron concentration was calculated. Neutron sensitizing activity of UTX-117, UTX-118 and BPA-F to B16-F10 cells were evaluated by WST-1 assay.



UTX-117



UTX-118

Fig.1

RESULTS: Cytotoxicity tests were performed by WST-1 assay using B16-F10 cells. As shown in Table 1, UTX-117 andUTX-118 exhibited hypoxia-selective cytotoxicity similar to that of tirapazamine.

Table. 1. Cytotoxicity of UTX-117 and UTX-118.

	Normoxia	Hypoxia	N/H ratio
UTX-117	>1000 μM	641.6 μM	>1.56
UTX-118	96.0 μM	33.8 μM	2.84
TPZ	123.2 μM	35.5 μM	3.74

N/H ratio=the IC_{50} value of Normoxia / Hypoxia

The cell uptake test of UTX-117 and UTX-118 was performed using B16-F10 cells (Fig. 2). The maximum uptake of BPA-F, a conventional BNCT agent, was approximately 1.3×10^9 atoms / cell [1]. On the other hand, the maximum uptake of UTX-118 was approximately 7.4×10^9 atoms / cell, it showed higher uptake efficiency than BPA-F. Since the boron concentration required for BNCT is at least 1.0×10^9 atoms / cell, a neutron sensitization effect can be expected for UTX-118.

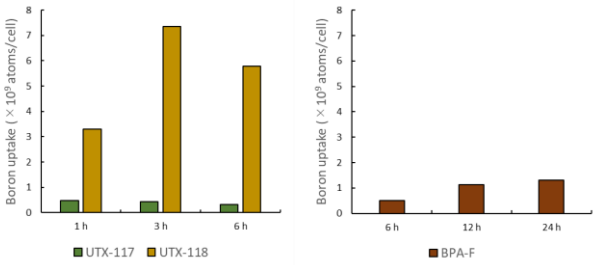


Fig. 2. Intracellular uptake of UTX-117 and UTX-118.

Neutron sensitizing activities of UTX-117, UTX-118 and BPA-F were evaluated using B16-F10 cells (Fig. 3). UTX-117 and UTX-118 showed the neutron sensitizing effect similar to BPA-F.

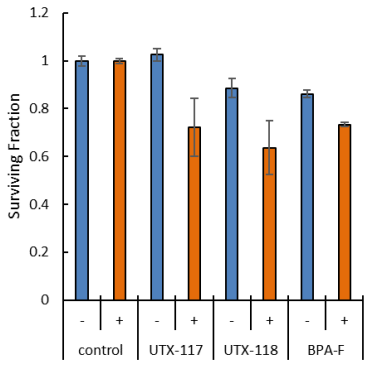


Fig. 3. Neutron sensitizing activity of UTX-117 and UTX-118. +: Irradiation, -: Non-irradiation.

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30P8-7

PR8-7 X-irradiation-induced bystander effect enhances invasion in tumor cells

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INTRODUCTION: In cancer studies, it has been suggested that irradiation sometimes enhances invasion of tumor cells. Wild-Bode et al. reported that sublethal dose of γ-ray irradiation enhanced invasiveness of human glioblastoma, and the enhancement was accompanied by the activation and/or up-regulation of integrin and matrix metalloproteinase (MMP) contributed to cellular adhesion and degradation of extracellular matrix (ECM). Fujita et al. also reported that X-ray irradiation enhanced cellular invasive ability via up-regulation of MMP in some human pancreatic tumors. Including these reports, a number of studies suggested that ECM degradation activity was involved in the irradiation-enhanced invasiveness of tumor cells. However, although there are many studies that estimate effects of direct irradiation on tumor cell invasiveness, not so many reports did bystander effects of irradiated tumor cell conditioned medium (CM) on it. Furthermore, the same effect of high LET radiation therapy such as boron neutron capture reaction (BNCT) has not been reported. In this study, we conducted the experiments to examine effects of X-irradiated tumor cell CM on cellular invasiveness in breast cancer and lung cancer-derived cells, prior to BNCT challenge.

EXPERIMENTS: Human breast adenocarcinoma MDA-MB-231 cells and human lung adenocarcinoma A549 cells were seeded on 60 mm dishes (1×10^6 cells/dish), and incubated with RPMI1640/10% FBS overnight. After washing by PBS, serum-free RPMI1640 medium was added to the dishes followed by 0, 2 or 4 Gy X-irradiation immediately. After 24 h incubation, the media were centrifuged for 5 min at 3,000 rpm at 4°C, and supernatants were collected as conditioned media (CM). Hereinafter, CM derived from 0, 2 and 4 Gy X-irradiated cells are described as 0 Gy CM, 2 Gy CM and 4 Gy CM.

Cellular invasive ability was evaluated using a BD BioCoat™ Matrigel™ Invasion Chamber (8 μm pore size, 24-well plate, BD Biosciences, Billerica, MA). Cells (2×10^4 MDA-MB-231 cells or 1×10^5 A549 cells) were suspended in 500 μl of serum-free RPMI1640 medium with or without 500 nM AG1478, and they were loaded into the upper chamber, followed by an addition of 750 μl of CM containing 1% FBS (CM/1 % FBS) into the lower chamber. After incubation for 6 h (MDA-MB-231 cells) or 12 h (A549 cells), non-invading cells were removed from the upper side of the membrane using cotton tips. Invading cells on the lower side of the membrane were fixed with 100% methanol and stained with 1% toluidine blue and 1% sodium borate. All the invading cells were counted using a light microscope. Invasion index was calculated as a relative value which set the number of 0 Gy CM-treated invaded cells to 1.

Cells (1×10^4 cells) were suspended with 100 μl of RPMI1640/10% FBS, and loaded into each well of a 96-well plate. After overnight incubation, the medium was exchanged with CM/1% FBS, and the cells were incubated for 6 h (MDA-MB-231 cells) or 12 h (A549 cells). Ten μl of WST-1 solution (3.24 μg/μl WST-1, 70 ng/μl PMS, 20 mM HEPES-NaOH [pH 7.4]) was added into each well, and 1 h incubation was performed. Absorbance (450 nm) of the each well was measured with Model 680 Microplate Reader (Bio-Rad Laboratories). Cellular proliferation activity was described as relative values which set the absorbance of 0 Gy CM-treated cells to 1.

RESULTS: In order to investigate the influence of CM on the infiltration ability of cells, an invasion assay was first performed. The lower chamber was filled with CM from MDA-MB-231 cells and A549 cells, and the upper chamber was filled with cells to evaluate the infiltration ability of each cell at 6 h and 12 h. As shown in Fig. 1, CM promoted the respective cell infiltration ability in a dose dependent manner of the irradiated X-rays. Next, a colorimetric test using WST-1 was performed to evaluate the effect of CM on cell proliferation activity. When MDA-MB-231 cells are treated with CM from X-irradiated cells for 6 h, 2 Gy CM and 4 Gy CM reduces cell proliferation activity about 2% and 9% as compared to 0 Gy CM. When A549 cells were treated with CM from X-irradiated cells for 12 h, 2 Gy CM and 4 Gy CM reduces cell proliferation activity about 3% and 24% as compared to 0 Gy CM. From these results, it was suggested that the X-ray irradiated cell-derived CM promotes cell infiltration ability even if taken into consideration cell proliferation activity. In next fiscal year, we plan to examine the effect of CM obtained after BNCT-treated cells.

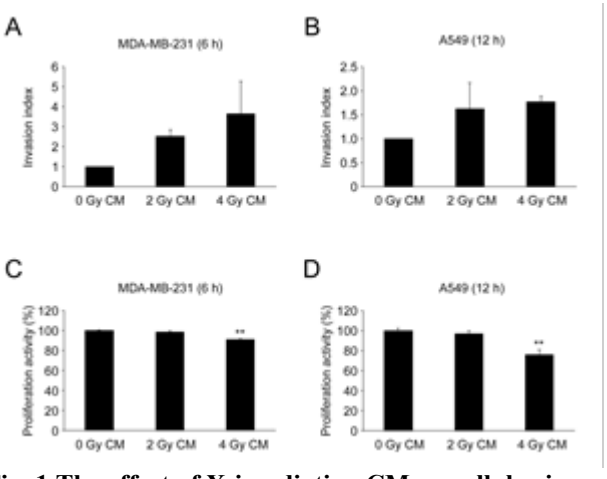

Fig. 1 The effect of X-irradiation CM on cellular invasion and proliferation.

PR8-8 Analysis of the response of tumor and normal tissues to BNCT

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INTRODUCTION: Boron neutron capture therapy (BNCT) is one of the particle beam radiation therapy based on the boron neutron capture fission reaction, which causes α particle and lithium nuclei of high linear-energy-transfer in a short range that is less than the diameter of typical cells. Boron compounds such as ^{10}B-boronophenylalanine (BPA) are mainly clinically used with neutron sources of nuclear reactors or accelerator-based BNCT systems. The cell death following DNA damage that is induced by BNCT[1)] is affected by various factors including the uptake of boron compounds by tumor cells and the thermal neutron fluence[2)]. Gamma-H2AX, a marker for DNA double strand breaks and poly(ADP-ribose) became high levels 6 or 24 hrs after boron neutron capture reaction (BNCR) in the rat lymphosarcoma model[3)]. The comprehensive analysis of the transcriptome and proteome after BNCR in human squamous carcinoma SAS cells has been carried out[4)]. As one of the factors induced after BNCR conditions, we focused on *CSF2* gene product, granulocyte-macrophage colony stimulating factor (GM-CSF). We studied the dynamic properties of GM-CSF and its mRNA (*CSF2*) after BNCR in cells and also in xenograft models of mice.

EXPERIMENTS: We used human squamous cell carcinoma-derived SAS and malignant melanoma-derived A375 cells. The neutron beam irradiation was operated at 1 MW at the Heavy Water Neutron Irradiation Facility of KUR reactor. Suspended cells were irradiated 2 hrs after incubation in the presence or absence of BPA 25 ppm (boron concentration) on Nov. 28th & Dec. 4th, 2018. For the xenograft models, SAS and A375 cells were transplanted into left hind legs of BALB/c nude male mice of 5 weeks old. Ten days later, neutron beam irradiation was carried out. As a boron compound, ^{10}B-BPA fructose complex was prepared before irradiation. Mice were irradiated approximately 30 min after intraperitoneal administration on Dec. 4th, 2018. Gold foil activation analysis was used for the measurement of thermal neutron fluences and thermoluminescence dosimeter (TLD) was used for the measurement of the γ-ray dose including secondary γ-ray. As previously described, total absorbed dose calculation was carried out using the flux-to-dose conversion factor by the sum of the absorbed doses resulting from ^{1}H(n, γ)^{2}D, ^{14}N(n, p)^{14}C, and ^{10}B(n, α)^{7}Li reactions. The cellular responses including factors present in culture supernatants were analyzed 6 and 24 hrs after irradiation of therapeutic doses of BNCT and γ-ray. Cell survival was analyzed by colony formation assay. The cells were transfected with siRNA of *CSF2* & *CSF2 receptor* using Lipofectamine™ 3000 Reagents (Thermo Fisher Scientific). GM-CSF levels in culture supernatant and mouse blood were measured using ELISA.

RESULTS: The irradiated beam and total radiation doses for cell experiments were exemplified in Table 1. SAS and A375 cells showed an increase in *CSF2* mRNA expression after the therapeutic dose irradiation of neutron beam with BPA, respectively. Growth of tumors derived from SAS cells was decreased 3 and 8 days after neutron beam irradiation in the presence of BPA compared with non-irradiated controls. Three days after irradiation, the DNA double strand break marker was positive in the tumors but not in the surrounding normal tissues. Changes in GM-CSF and other factors in the blood of xenograft models and cell culture are being analyzed. The results suggest that CSF2 and CSF2 receptor pathway is limitedly involved in the cancer cell survival. The dynamic changes of GM-CSF levels in tumors suggest its involvement in the regulation of local tumor microenvironment after BNCR and this point is being studied further.

Table 1. The irradiated thermal neutrons and doses.

Irradiation time (min)	Position	Thermal neutron fluence (/cm^2)	Total dose (Gy-Eq)	Average dose (Gy-Eq)
10	Sample surface	1.3E+12	2.9	2.2
	Back side	6.2E+11	1.5	
60	Sample surface	7.1E+12	9.3	12.1
	Back side	3.6E+12	14.9	
7	Sample surface	7.1E+11	1.6	1.4
	Back side	5.4E+11	1.3	
12	Sample surface	1.2E+12	2.6	2.3
	Back side	8.9E+11	2.0	
36	Sample surface	3.7E+12	8.3	7.4
	Back side	2.8E+12	6.4	

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[4] A. Sato *et al.*, Appl. Rad. Iso., **106** (2015) 213-219.

PR8-9 Radiobiological effect of extratumoral boron distribution and Neutron irradiation

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INTRODUCTION: Boron Neutron Capture Therapy (BNCT) is a particle radiation therapy for malignant diseases. However, Boron distribution of extra-cellular fluid or interstitial tumor tissue during the neutron irradiation and radiobiological effect of extracellular born neutron reaction is still unclear. In the previous studies, we have focused on intra-cellular boron concentration and tumor tissue boron concentrations. These were special average boron concentration and there have intrinsic heterogeneity. The goal of this study is, to clarify a role of extra-cellular / peri-tumoral boron neutron reaction in BNCT.

EXPERIMENTS:

Materials

Boron compounds were prepared using previously published method [1]. *p*-boronophenylalanine (BPA) were purchased from Interpharma Ltd. (Praha, Czech Republic). Fructose-BSH final concentration was 2400μg/mL ^{10}B.

Cell Lines

CT-26 murine colon cancer cell lines were cultured in D-MEM supplemented with 10% fetal bovine serum and maintained at 37℃ in a humidified atmosphere with 5% CO_2. After trypsinized and counted, cells were suspended in culture medium.

Tumor models

4w female bulb/c mice 1.0x10^{7}cells were implanted to rt. thigh subcutaneously.

Boron Neutron Reaction

BPA was administrated via tail vain or i.p. injection 2hrs before neutron irradiation. Thermal neutron dose was 7.3x10^{12} n/cm^2.

Tumor progression were measured by tumor size in each group.

RESULTS AND DISCUSSIONS:

As shown in Fig. 1, The group which administrated via i.p. has more inhibitory effect than i.v. group. These two groups were administrated the same dose, some boron agent. It is presumed that the area under curve of BPA during neutron irradiation is the difference. It is easily conceivable that the peak boron concentration of i.v. group reached just after administration and blood boron concentration was rapidly reduced by renal excretion. On the other hand, i.p. group reached peak boron concentration of i.p. group reached after a certain period, and supplied from interstitial tissue to blood continuously, then reduced moderately.

Neutron irradiation without boron administration group also showed inhibitory effect. With or without boron, these inhibitory effects were showed as a reducing growth curve rate. It means there are sublethal damaged cells despite high LET radiation, or damaged tumor surrounding normal tissue which supplying a proliferation factor or a nutrient factor to tumor. and such inhibitory effect prolonged after single boron neutron reaction. Ono [2] advocated that the tumor cell density connect with BNCT effectiveness.

To clarify this inference, the detailed boron distribution of intra and extra tumor cells, and interstitial peritumoral tissue should be examined.

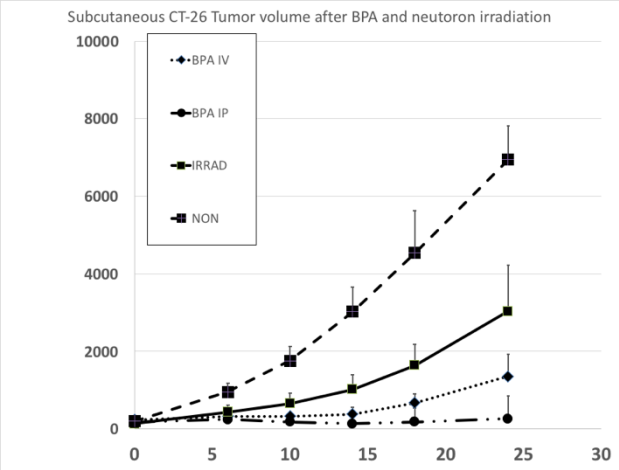

Fig. 1. BNCT treatment of subcutaneous model of colon cancer. Three groups of animals underwent the irradiation treatment at KUR. The first group (irradiated and i.v. treatment group, n=6) received f-BPA at a dose of 250mg^{10}B/kg, i.v. via tail vein 2 hours before neutron exposure. The second mice group (irradiated and i.p. treatment group, n=5) received the same volume of f-BPA i.p. 2hrs before the irradiation. The third group (irradiated only, n=6) received neutron exposure only. Nine mice were followed up as non-treatment group.

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30P8-10

PR8-10 Multilateral Approach toward Realization of Next Generation Boron Neutron Capture Therapy

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[3]*CyDing Co., Ltd*
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INTRODUCTION: Boron neutron capture therapy (BNCT) is a next-generation radiation therapy that irradiates thermal neutrons to boron compounds accumulated in tumor cells and selectively irradiates tumor cells with high LET radiation by the generated α-rays and Li nuclei. The therapeutic effect of BNCT greatly depends on the boron compound which collects boron at the tumor site, and existing boron compounds, L-p-Boronophenylalanine (L-BPA) and disodium mercaptoundecahydrododecaborate (BSH) have limitation with regard to the adaptive cancer type and its sufficient and specific accumulation to tumor. Therefore, new boron compounds and carrier with higher tumor cell accumulation without normal tissue accumulation are being searched. BSH don't have the active accumulation to tumor cells, but it has 12 x 10B in one molecule, BSH induces a strong biological effect even with small accumulation. Folate receptor-α (FR-α) is highly expressed on the many tumor (ovarian, kidney, colorectal, et al.), and it is useful as a target for drug delivery system (DDS) against cancer. It has been reported that the compound which is a cyclic oligosaccharide cyclodextrin modified with folic acid, has improved tumor accumulation and therapeutic effects of paclitaxel (PTX) and doxorubicin (DOX), which are anticancer drugs. In this study, we aimed to construct BSH inclusions with folate-modified cyclodextrin (ND 201) and to realize active accumulation of BSH against folate-targeted tumor and its usefulness.

EXPERIMENTS: Colon-26 cells derived from murine colorectal cancer and A549 cells derived from human lung cancer were purchased from RIKEN BioResource Research Center. Colon-26 cells show the overexpression of FR and A549 cells show low expression level of FR. BALB/c nu/nu mice were used for in vivo kinetics experiments. BSH was purchased from Stella Pharma in powder form and dissolved in a phosphate buffer at the appropriate time before the experiment. ND201 was purchased from NanoDex corporation and dissolved in 0.1 mol/l carbonic acid/bicarbonate buffer (pH9-10). The solution was neutralized with a phosphate buffer (pH 6.8-7.2) and stocked at -30°C freezer. The interaction between BSH and ND201 was evaluated from stability constants and stoichiometric ratio. BSH and BSH containing ND201 (BSH-ND201) were administered from the tail vein to mice at concentrations of 100 and 5 mg/kg, respectively. Boron concentration (ppm) in the tumor and blood was measured with ICPS-8100 (Shimadzu Corporation) and the tumor/blood (T/B) ratio was calculated with each value.

RESULTS: The stability constants Kc was 1.4×104 (/M) in BSH and the value suggests that ND201 and BSH shows stable complex in serum-containing culture medium and human blood. The stoichiometry of a host-guest complex was determined by the continuous variation plot method. The plots made by monitoring the fluorescence intensity change gave a maximum peak at 0.5, indicating that ND201 forms an inclusion complex with BSH at a 1:1 molar ratio. Next, the boron concentration in tumors and blood of BALB/c nu/nu mice was measured by ICP-MS. The concentration in blood showed similar time course kinetics after BSH and BSH-ND201 without depending on the tumor type. On the other hand, the concentration in Colon-26 tumor showed drastic decrease immediately after BSH administration, whereas it increased to 24 hours and showed high value at 72 hours after BSH-ND201 administration. The T/B ratio when the intratumoral boron concentration was peak was calculated and BSH-ND201 showed high T/B ratio (10.6) for Colon-26 tumor, and this value satisfied the T/B ratio > 10 required for clinical safety in BNCT. On the other hand, the ratio was too low (1.6) for A549 tumor.

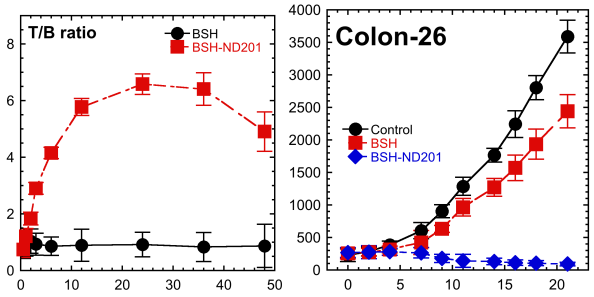

Fig. 1. Comparison of the T/B ratio and anti-tumor effects using BSH and novel boron complex, BSH-ND201.

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PR8-11 Attempts to sensitize tumor cells by exploiting the tumor microenvironment

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INTRODUCTION: Hypoxia and glucose deprivation have been suggested to play important roles in resistance to radiation [1]. Attempts to sensitize tumor cells by exploiting the tumor microenvironment have been studied. A major mediator of the cellular hypoxic response, hypoxia inducible factor 1 (HIF-1), is a potential target for cancer therapy, because it transcriptionally regulates a number of genes, including those involved in glucose metabolism, angiogenesis and resistance to chemotherapy and radiation therapy [2]. We previously reported that the disruption of Hif-1α enhanced the sensitivity of murine squamous cell carcinoma (SCC-VII) cells to gamma-ray [3]. In the present study, we investigated whether the disruption of Hif-1α affects the radiosensitivity of SCC-VII cells to the boron neutron capture reaction (BNCR).

EXPERIMENTS: In vitro, SCC-VII and SCC-VII-Hif-1α-deficient (ΔHif-1α) cell suspensions were incubated with 10B-carrier (BPA or BSH) at 20 ppm 2h before neutron irradiation, and cell survival assay was performed.
In order to examine the effect of hypoxia on cell survival, the cells were cultured for 12 h under normoxic or hypoxic (1% O2) condition, and then incubated with 10B-carrier.
In order to examine the influence of the disruption of Hif-1α in vivo, SCC-VII or SCC-VIIΔHif-1α cells were inoculated subcutaneously into the hind legs of C57BL/6 mice. Tumor-bearing mice were irradiated with a reactor neutron beam, and then clonogenic cell survival assay. Irradiation was started from 60 min after the subcutaneous injection of 10B-carrier (BPA 250 mg/kg or BSH 125 mg/kg).

RESULTS: We examined the cell survival fraction of SCC-VII or SCC-VII-ΔHif-1α cells irradiated with the neutron beam. In the presence of BSH (data not shown) or BPA (Fig. 1), the cell survival after neutron irradiation was lowered. Moreover, SCC-VII-ΔHif-1α cells with BPA exhibited lower survival compared to SCC-VII cells with BPA (Fig. 1). We also investigated the influence of oxygen levels on cell survival. In the presence of BPA, SCC-VII cells (hypoxia) showed more resistant to the neutron beam than SCC-VII cells (normoxia), similar to the previous research [1]. However, there was no significant difference in survival of the ΔHif-1α cells between hypoxia and normoxia groups.
In order to examine the influence of the disruption of Hif-1α in vivo, tumor-bearing mice were irradiated with a reactor neutron beam. As shown in Fig. 2, in the absence of BSH or BPA, SCC-VII and SCC-VII-ΔHif-1α cells from tumor-bearing mice showed similar survival fraction. In the BSH-administrated group, ΔHif-1α cells exhibited higher survival than the SCC VII cells. In the BPA-administrated group, ΔHif-1α cells was more sensitive than the SCC VII cells.

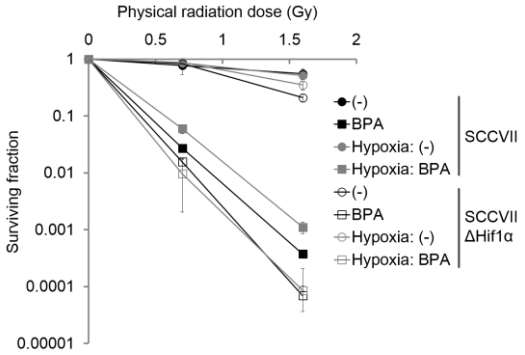

Fig. 1 Cell survival curves for SCC VII or SCC-VII ΔHif-1α cells.

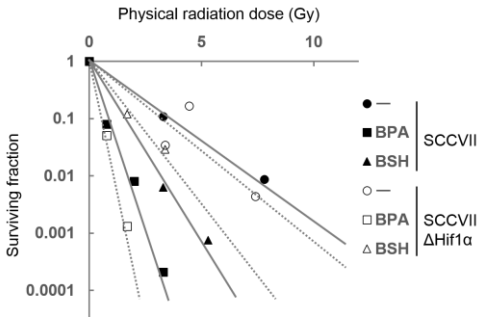

Fig. 2 Cell survival curves for the total cell population from SCC VII or SCC-VII ΔHif-1α tumors irradiated with neutron beam.

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30P8-12