VIII-Ⅱ-1. Project Research

Project 4

PR4 Analyzing Tumor Microenvironment and Exploiting its Characteristics for Controlling Malignant Solid Tumors and Distant Metastatic Potential

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BACKGROUNDS AND PURPOSES: Human solid tumors are thought to 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 that have been employed for various oncology studies [1]. 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 [1]. 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 [1]. 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 cell populations that could not be sufficiently killed by radiotherapy [1]. Further, sufficient doses of drugs cannot be distributed within Q tumor cell populations mainly due to heterogeneous and poor vascular distributions within solid tumors. Thus, one of the major causes of post-chemotherapy recurrent tumors is an insufficient dose distribution in 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 [2]. Q cells showed greater potentially lethal damage repair (PLDR) capacities than the total cells. γ-Ray irradiation and neutron irradiation with BPA induced larger PLDR capacities in each cell population. In contrast, thermal neutron irradiation without the ^{10}B-compound induced the smallest PLDR capacity in both cell populations. The use of the ^{10}B-compound, especially BPA, resulted in an increase in the PLDR capacity in both cell populations, and made the PLDR patterns of the both cell populations look like those induced by γ -ray irradiation [2]. In both the total and Q tumor cells, the hypoxic fractions (HFs) immediately after neutron irradiation increased suddenly. Reoxygenation after each neutron irradiation occurred more rapidly in the total cells than in the Q cells. In both cell populations, reoxygenation appeared to be rapidly induced in the following order: neutron irradiation without ^{10}B-compounds > neutron irradiation following BSH administration > neutron irradiation following BPA administration > γ -ray irradiation [2]. These findings concerning the difference in sensitivity, PLDR and reoxygenation following neutron irradiation after ^{10}B-compound administration were thought to be mainly based on the fact that it is difficult to deliver a therapeutic amount of ^{10}B from currently used ^{10}B-carriers throughout the target tumors, especially into intratumor hypoxic cells with low uptake capacities [3].

Therefore, 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 radiation therapy including BNCT in the use of newly-developed ^{10}B-compound based on the revealed findings on intratumor microenvironmental characteristics.

RESEARCH SUBJECTS:

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

ARS-1 (25P4-1): 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,Y. Sakurai, H. Tanaka, M. Takagaki, and H. Matsumoto)

ARS-2 (25P4-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers
(H. Nagasawa, S. Masunaga, K. Okuda, S. Y. Hirayama and A. Isono)

ARS-3 (25P4-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level
(H. Harada and S. Masunaga)

ARS-4 (25P4-4): Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction
(R. Hirayama, S. Masunaga, Y. Sakurai, H. Tanaka and Y. Matsumoto)

ARS-5 (25P4-5): Transdermal Drug Delivery System using Hyaluronan-Conjugated Liposomes as ^{10}B-Carrier in Boron Neutron Capture Therapy for Melanoma
(S. Kasaoka, K. Hashimoto and S. Masunaga)
Due to the irregular and short operation period of our reactor in 2013, the data from this research group could not be shown this time.

ARS-6 (25P4-6): Evaluation of Inclusion Complex of Carborane Modified Kojic Acid and Cyclodextrin as ^{10}B-Carrier in Boron Neutron Capture Therapy
(T. Nagasaki, S. Masunaga, M. Kirihata, H. Azuma, R. Kawasaki, M. Sakuramoto, Y. Hattori and N. Kadono)

ARS-7 (25P4-7): Molecular design and synthesis and functional evaluation of anticancer and molecular targetting agents
(Y. Uto, H. Hori, R. Tada and S, Masunaga)

ARS-8 (25P4-8): Analyzing biological effect of BNCT from the viewpoint of the changes in oxygenation level
(H. Yasui, S, Masunaga, O. Inanami, T. Yamamori and D. Nagane)

ARS-9 (25P4-9): Analyses on the Responsiveness of Malignant Tumors to BNCT.
(M. Masutani, S. Masunaga, H. Ito, S. Saito, H. Sakuma, M. Hirai, S. Nishio and H. Okamoto)

(Underline: Representative at each research group)

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[2] S. Masunaga *et al.*, J. Radiat. Res. **43** (2002) 11-25.
[3] S. Masunaga *et al.*, Clin. Exp. Metastasis **26** (2009) 693-7.

採択課題番号 25P4　局所腫瘍制御と転移抑制を同時に目指す治療法開発のための　プロジェクト
がん微小環境解析とその応用
（京大・原子炉）増永慎一郎

PR4-1 Dependency of Compound Biological Effectiveness (CBE) Factors on the Type and Concentration of Administered Neutron Capture Agents in BNCT

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BACKGROUNDS AND PURPOSES: The greater density of ionizations along high linear energy transfer (LET) particle tracks results in an increased biological effect compared with the same physical dose of low LET radiation. This is called relative biological effectiveness (RBE), which is the ratio of the absorbed dose of a reference source of radiation (e.g., X-rays) to that of the test radiation that produces the same biological effect. Because both tumor and surrounding normal tissues are present in the radiation field, there will be an unavoidable, nonspecific background dose, consisting of both high and low LET radiation. However, a greater concentration of ^{10}B in the tumor will result in receiving a much higher total dose than that of adjacent normal tissues. This is the basis for the therapeutic gain in BNCT. The total radiation dose delivered to any tissue can be expressed in photon equivalent units as the sum of each of the high LET dose components multiplied by weighting factors, which depend on the increased radiobiological effectiveness of each of these radiation components.

The dependence of the biological effect on the microdistribution of ^{10}B requires the use of a more appropriate term than RBE to define the biological effects through the ^{10}B(n,α)^{7}Li reaction. Measured biological effectiveness factors for the components of the dose from this reaction have been originally introduced by Gahbauer *et al*. as the "compound factors" [1]. Subsequently, the factors have been called "compound biological effectiveness (CBE) factors". The mode and route of the administration of a ^{10}B-carrier, the ^{10}B distribution within the tumor, normal tissues, and even more specifically within cells, and even the size of the nucleus within the target cell population, can all influence the experimental determination of the CBE factor. CBE factors are fundamentally different from the classically defined RBE, which is primarily dependent on the quality (i.e., LET) of the radiation administered. CBE factors are strongly influenced by the distribution of the specific ^{10}B delivery agent and can differ substantially, although they all describe the combined effects of α particles and ^{7}Li ions. The CBE factors for the ^{10}B component of the dose are specific for both the ^{10}B delivery agent and the tissue. A weighted Gy unit [Gy(w)] has been used to express the summation of all BNCT dose components and indicates that the appropriate RBE and CBE factors have been applied to the high LET dose components. However, for clinical BNCT, the overall calculation of photon equivalent [Gy(w)] doses requires several assumptions about RBEs, CBE factors, and the ^{10}B concentrations in various tissues, based on currently available human or experimental data [2].

Here, we tried to analyze the changes in the values of RBE for neutron only irradiation and CBE factors for employed ^{10}B-carriers according to their concentrations when administered *in vivo*. The neutron capture reaction was performed with two ^{10}B-carriers, boronophenyl- alanine-^{10}B (BPA) and sodium mercaptoundecahydro- dodecaborate-^{10}B (BSH). Regarding the local tumor response, the effect not only on the total (= proliferating (P) + quiescent (Q)) tumor cell population, but also on the Q cell population, was evaluated using our original method for selectively detecting the response of Q cells in solid tumors.

MATERIALS AND METHODS: After the subcutaneous administration of a ^{10}B-carrier, BPA or BSH, at 3 separate concentrations, the ^{10}B concentrations in the tumors were measured by γ-ray spectrometry. SCC VII tumor-bearing C3H/He mice received 5-bromo-2'- deoxyuridine (BrdU) continuously to label all intratumor P cells, then treated with BPA or BSH. Immediately after reactor neutron beam irradiation, during which intratumor ^{10}B concentrations were kept at levels similar to each other, cells from some tumors were isolated and incubated with a cytokinesis blocker. The responses of the Q and total (= P + Q) tumor cells were assessed based on the frequencies of micronucleation using immunofluorescence staining for BrdU.

RESULTS: The values of calculated CBE were higher in Q cells and in the use of BPA than total cells and BSH, respectively. In addition, the higher the administered concentrations were, the smaller the values of CBE became, with a more marked tendency in the use of BPA than BSH. The CBE values for neutron capture agents that deliver to solid tumors more dependently on intratumor heterogeneity become more changeable.

DISSCUSSION AND CONCLUSIONS: Tumor heterogeneity is one of the major difficulties in the treatment of solid tumors. Although a lot of research on tumor heterogeneity are now going on, there are not yet any apparent indices for evaluating the degree of intratumor microenvironmental heterogeneity. The CBE factor for each tissue and tumor, which greatly depends on the degree of the possibility for distributing ^{10}B from a ^{10}B-carrier, can be a promising candidate for the index for estimating tumor heterogeneity [3].

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[3] S. Masunaga, *et al*. Springer Plus **3** (2014) 128. doi:10.1186/2193-1801-3-128.

採択課題番号 25P4-1　腫瘍内各特定細胞集団の制御と転移制御をも目指す　プロジェクト
癌治療(特に BNCT)の最適化

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PR4-2 Design and Synthesis of a New Membrane Peameable Boron Carrier Using the Lipidated Peptide Vehicle

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INTRODUCTION: For the success of boron neutron capture therapy (BNCT), it is essential to selectively deliver sufficient amount of ^{10}B atoms to tumor cells. We have demonstrated that the unique mode of direct cell penetration of lipidated peptides called pepducins which were discovered as allosteric modulators of GPCRs [1]. Pepducins consist of a synthetic peptide derived from the appropriate intracellular loop domains of GPCRs, typically 10–20 amino acids in length, and a fatty acid such as palmitic acid. By the fluorescence resonance energy transfer (FRET)-based imaging of transmembrane movement of pepducins, it was shown that they directly penetrate cellular membrane not by endocytosis but by flip diffusion (Fig. 1) [2]. Then we envisage that membrane impermeable hydrophilic molecules could be delivered into cytosol by means of pepducin as a transmembrane delivery vehicle.

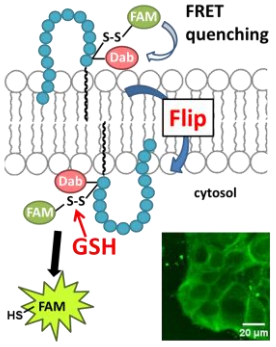
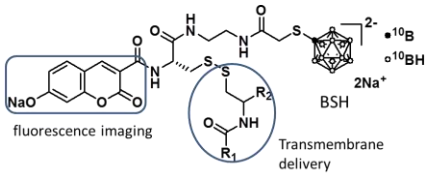


Fig. 1 Transmembrane movement of the FRET probe of pepducin, Pep-Dab-SS-FAM. FAM was released into cytosol by GSH reduction.

To develop a tumor selective boron carrier, we developed a membrane permeable boron carrier in the first stage. We evaluated here the structural requirement of the pepducin moiety for the direct transmembrane delivery by live cell fluorescence imaging.

EXPERIMENTS:
As shown in Fig. 2, we designed and synthesized new pepducin vehicle loaded with BSH, Pep-SS-Cou-BSH for the intracellular delivery of membrane impermeable anionic boron claster BSH. Coumarin fluorophore is attached to monitor the cellular distribution.

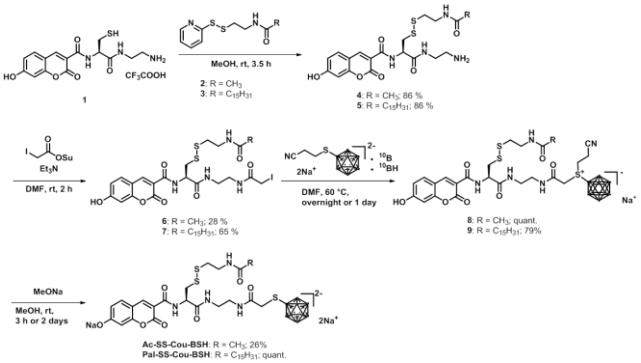


Ac-SS-Cou-BSH (R_1 = CH_3, R_2 = H)
Pal-SS-Cou-BSH (R_1 = $C_{15}H_{31}$, R_2 = H)
Pep-SS-Cou-BSH(R_1 = $C_{15}H_{31}$, R_2 = peptide)

Fig. 2 Design and synthesis of membrane penetrating boron carrier

To evaluate the function of lipid moiety and peptide respectively, control probes, Ac-SS-Cou-BSH and Pal-SS-Cou-BSH ware also synthesized. We performed live cell imaging experiments using MCF-7 cells with the probes and confocal microscopy.

RESULTS: Acetyl and palmitoyl derivatives were obtained as shown in Scheme 1.



Scheme 1 Syntheses of As-SS-Cou-BSH and Pal-SS-Cou-BSH

By the treatment with Pal-SS-Cou-BSH, a fluorescence signal was observed along the outline of the cell, which suggested that the probe was localized on the outer-leaflet of a cell plasma membrane. On the other hand, no fluorescence signal was observed treated with Ac-SS-Cou-BSH, and it was thought that it was washed away without delivery to cell surface (Fig. 3).

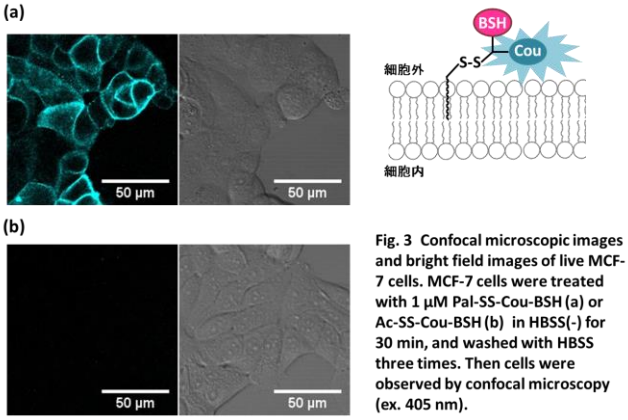


Fig. 3 Confocal microscopic images and bright field images of live MCF-7 cells. MCF-7 cells were treated with 1 μM Pal-SS-Cou-BSH (a) or Ac-SS-Cou-BSH (b) in HBSS(-) for 30 min, and washed with HBSS three times. Then cells were observed by confocal microscopy (ex. 405 nm).

CONCLUSION: These data show lipid moiety is necessary to anchor the probe in membrane. Now, we are examining intracellular delivery of Pep-SS-Cou-BSH by fluorescence imaging.

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採択課題番号 25P4-2　低酸素微小環境を標的とするボロンキャリアの開発　プロジェクト
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PR4-3 IDH3α Overexpression Promotes Malignant Tumor Growth by Inducing HIF-1 Activity

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INTRODUCTION: Metastasis causes death among cancer patients. Accumulating evidence has shown that hypoxia-inducible factor 1 (HIF-1) plays critical roles in distant tumor metastases at multiple steps. Clinical studies have also demonstrated that HIF-1 could be used as an adverse prognostic factor for local tumor recurrence and distant tumor metastasis in cancer patients. These findings justify targeting HIF-1 for cancer therapies.

HIF-1 is a heterodimer composed of an α-subunit (HIF-1α) and a β-subunit (HIF-1β) and its activity is mainly dependent on stability and trans-activating activity of HIF-1α. Both of them are strictly regulated by α-ketoglutarate (KG)-dependent enzymes, PHDs and FIH-1, respectively. PHDs hydroxylate proline residues, P402 and P564, in the oxygen-dependent degradation domain (ODD domain) of HIF-1α in both oxygen- and α-KG-dependent manner. The hydroxylations trigger polyubiquitilation and subsequent proteasomal degradation of HIF-1α. FIH-1 hydroylates asparagine residue, N803, in the C-terminal transactivation domain (C-TAD) of HIF-1α, resulting in the suppression of its transactivating activity under normoxic conditions. On the contrary, HIF-1α becomes active under hypoxic conditions because of the inactivation of these hydroxylases, and then, interacts with its binding partner, HIF-1β. Resultant heterodimer, HIF-1, binds to its cognate enhancer sequence, the hypoxia-responsive element (HRE), and induces transcriptions of various genes related to not only the escape from hypoxia (invasion and metastasis of cancer cells) but also the improvement of oxygen-availability (angiogenesis), and adaptation of cellular metabolism to hypoxia (metabolic reprogramming).

In order to explore novel genes which are responsible for the activation of HIF-1, we had established a new screening method and found that overexpression of wild-type IDH3α is responsible for the activation of HIF-1. Because last year we elucidated molecular mechanism underlying the IDH3α-mediated activation of HIF-1 (IDH3 overexpression decreases intracellular levels of α-KG and resultantly stabilizes HIF-1α protein), this year we analyzed whether investigated whether the positive impact of IDH3α on the stabilization and activation of HIF-1α influenced tumor growth.

EXPERIMENTS & RESULTS: We established stable transfectants of HeLa cells with the IDH3α expression vector (HeLa/IDH3α) or its empty vector (HeLa/EV). The overexpression of IDH3α significantly accelerated the growth of HeLa/IDH3α tumors xenografted in immunodeficient mice; however, the positive effect was not very prominent, which may have been because the stable transfectants expressed particular levels of endogenous IDH3α. Thus, we performed loss-of-function studies by silencing endogenous IDH3α to directly examine the involvement of IDH3α in tumor growth. HeLa cells were stably transfected with plasmids expressing short hairpin RNAs against the IDH3α sequence (shIDH3α) and scramble sequence (Scr) to establish stable IDH3α knockdown cell lines (HeLa/shIDH3α cell lines) and their control counterparts (HeLa/Scr cell lines), respectively. We confirmed that the knockdown of IDH3α markedly suppressed the induction of HIF-1α expression in HeLa/shIDH3α cells. Stable knockdown significantly delayed the growth of tumor xenografts *in vivo*. These results indicated that the IDH3α-mediated activation of HIF-1 in cancer cells enhanced the growth of malignant tumors, and therefore, can be exploited as a rational target for cancer therapy.

We analyzed associations between IDH3α expression levels and prognosis in human lung and breast cancers using the PrognoScan database to validate our results in human tumors. We confirmed that IDH3α expression levels were positively correlated with poor overall survival in various human cancers, such as breast and lung cancers. RefEx analysis, which enables the expression levels of any interesting genes to be quantified in 10 major groups of normal tissues based on a public dataset of microarrays (GeneChip), revealed that the expression levels of IDH3α in normal tissues were lower than those of VEGF-A, one of the most important target molecules for cancer therapy. These results highlighted the importance of IDH3α expression levels as a prognostic marker as well as a therapeutic target.

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採択課題番号25P4-3　低酸素誘導性因子1(HIF-1)を活性化する新規遺伝子の探索と プロジェクト機能解析、および局所腫瘍制御への展開
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PR4-4 Hydroxyl Radical-mediated Indirect Actions of X-ray-induced Cellular Lethality are the Major Cause for the Large Oxygen Enhancement Effect

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INTRODUCTION: Over the past few years, we published studies on the role of free radicals in the biological response to heavy-ion radiation [1-3]. As is well established, X-rays induce DNA damage via two interacting mechanisms: Indirect actions are mediated by radicals arising from water radiolysis, while direct actions are due to the deposition of radiation energy on DNA. Hydroxyl radical (•OH) is one of the main radiolysis products in irradiated water, which accounts for a large fraction of the indirect actions, although the generation of other reactive oxygen species such as O_2^- and H_2O_2 and the following reactions are shown.

EXPERIMENTS: *Cells culture*: The Chinese hamster ovary cell lines, CHO and AA8, were provided by the RIKEN BRC. These two cell lines have been used extensively to study DNA repair in wild-type cells. The cells were grown in Ham's F12 medium (SIGMA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under a humidified air with 5% carbon dioxide at 37˚C. The cells were rinsed in calcium- and magnesium-free phosphate-buffered saline (PBS), harvested with 0.05% trypsin-EDTA solution (SIGMA) and seeded into 45-mm diameter glass dishes at a concentration of 2 × 105 cells per dish. CHO and AA8 cells were cultured for 24 or 48 hours prior to irradiation, respectively. *Irradiation and treatment with DMSO in oxic and hypoxic conditions*: X-rays were produced by an X-ray generator (SHIMADZU, PANTAC HF-320S) with a tungsten target, operated at 200 kV and 20 mA with 0.5 mm-aluminum and 0.5 mm-copper filters. The X-rays were delivered to the samples at room temperature at a dose rate of 4.9 Gy/min, which was determined by Fricke dosimetry. The glass dishes were filled with 1.2 ml of medium alone or containing different concentrations of DMSO ranging from 0 to 1.0 M and then transferred to an irradiation chamber. For exposure under oxic and hypoxic conditions, the chambers were flushed for more than 1 hour before irradiation with a gas mixture consisting of 1000 ml/min of 95% air and 5% pure carbon dioxide or 95% pure nitrogen and 5% pure carbon dioxide, respectively. These gases were passed through a bubbling bottle to maintain high humidity levels before flowing into the chambers. The pO2 level in the hypoxia medium was measured using an oxygen electrode as described previously [3] and the level was consistently found to be less than 0.2 mmHg (0.026%). *Colony formation assay*: After irradiation, the cells were rinsed twice with PBS, harvested with 0.05% trypsin-EDTA solution and re-suspended in 1 ml of fresh medium for cell number counting. CHO and AA8 cells were seeded into three replicate 60-mm diameter plastic dishes at a density of approximately 100 living cells per dish in 5 ml of growth media and incubated for 12 or 13 days, respectively. The colonies were fixed with 10% formalin in PBS for 10 min, stained with 1% methylene blue in water and colonies consisting of more than 50 cells were counted. The 10% survival level (D_{10} or LD_{90}) was calculated from a dose-response curve fitted by a linear-quadratic (LQ) equation. *Calculation of the maximum protection by DMSO*: The maximum degree of protection (DP), the concentration of DMSO that provides the maximum protection against cell killing, was calculated following our previous work [1]. Briefly, the maximum DP was calculated by an extrapolation of reciprocals of surviving fractions over those of DMSO concentrations. To calculate DPs under oxic and hypoxic conditions, we used the data points beyond the shoulder region of the survival curves where the curves decreased exponentially. Regression curves were drawn in the plots of DP as a function of DMSO concentration.

RESULTS: The contributions of indirect actions on cell killing of CHO cells were 76% and 50% under oxic and hypoxic conditions, respectively, and those for AA8 cells were 85% and 47%, respectively. Thus, the indirect actions on cell killing were enhanced by oxygen during X-irradiation in both cell lines tested. Oxygen enhancement ratios (OERs) at the 10% survival level (D_{10} or LD_{90}) for CHO and AA8 cells were 2.68 ± 0.15 and 2.76 ± 0.08, respectively. OERs were evaluated separately for indirect and direct actions which gave the values of 3.75 and 2.01 for CHO, and 4.11 and 1.32 for AA8 cells, respectively. Thus the generally accepted OER value of around 3 is best understood as the average of the OER values for both indirect and direct actions. These results imply that both indirect and direct actions on cell killing require oxygen for the majority of lethal DNA damage. However, oxygen plays a larger role for the former than for the latter. On the other hand, the lethal damages induced by the direct actions of X-rays are less affected by oxygen concentration.

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採択課題番号 25P4-4　中性子捕捉反応における細胞致死機構の放射線作用解析　プロジェクト
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PR4-5 Hypoxia-Induced Factor (HIF)-1α Inhibition and Metastasis Suppression with BNCT by Using Kojic Acid Modified *o*-Carborane

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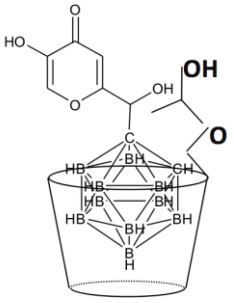
INTRODUCTION: Treatment of metastatic malignant melanoma remains ongoing serious challenges. Boron neutron capture therapy (BNCT) is single cell-selective radiation therapy. Therefore, BNCT has been attracted great deal of attention as a potent modality for malignant melanoma. The success of BNCT depends on the boron delivery system to accumulate effectively and deeply inside the tumor cells. We previously reported that Kojic acid-modified *o*-carborane conjugate (CKA) showed melanoma cells selectivity, unique nuclear localization, and high tumor-suppression effect on BNCT toward melanoma-bearing mice. Herein, mechanism of high efficiency on BNCT and suppression effect on metastasis is evaluated.

EXPERIMENTS: Since CKA is poorly water-soluble compound, the inclusion complex of hydroxypropyl-β-cyclodextrin (HP-β-CD) was evaluated as boron drug for melanoma-targeting BNCT. The antitumor efficiency of CKA/HP-β-CD complex were evaluated by using tumor-bearing mice implanted with B16BL6 cells. CKA/HP-β-CD and L-BPA fructose complex were injected by intraperitoneal administration at doses of 10 mg ^{10}B /kg (1000 ppm of ^{10}B concentratin; 200 μl) before 1 hr of irradiation. Neutron irradiation was carried out at Kyoto University Research Reactor (5 MW, 18 min, 5.0×10^{12} neutron/cm^2). After 14 or 21 days, the mice were sacrificed and autopsied to count metastatic colonies in the bilateral lungs. In order to estimate the effect of CKA/HP-β-CD complex on hypoxia-induced accumulation of HIF-1α protein, HeLa cells were incubated for 5 h with or without the indicated concentrations of compound under 1% oxygen hypoxic condition. The levels of protein were detected by immunoblot analysis with HIF-1α protein or Hsp90 specific antibodies. Hsp90 was used as the control.



Structure of CKA/HP-b-CD

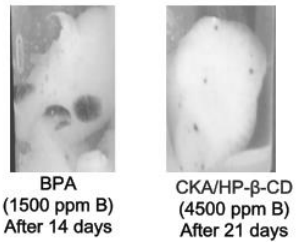


Fig. 1. Inhibition effect of CKA complex on metastasis to lung after BNCT.

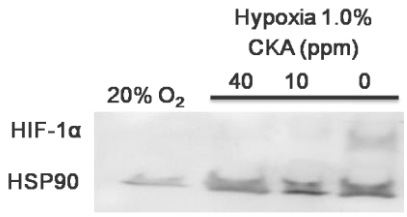


Fig.2. Inhibition effect of CKA complex on hypoxia-induced accumulation of HIF-1α in HeLa cells.

RESULTS AND DISCUSSION: By irradiation, proliferation and antitumor efficiency of BNCT were improved within concentration-dependent and neutron fluence-dependent (data not shown). Moreover, CKA/HP-β-CD complex shows not only similar or superior tumor suppression effect to L-BPA but also metastatis suppression effect (Fig. 1). K. Shimizu et al, reported that *o*-carborane-containig phenoxyacetanilide derivatives showed strong inhibition effect on hypoxia-induced HIF-1α accumulation [1]. HIF-1 is well known as the transcription activator ofproteins involving invasion and metastasis [2]. We so examined the effects of CKA/HP-β-CD complex against hypoxia-induced HIF-1α accumulation by western blot analysis inHeLa cells. CKA/HP-β-CD complex suppressed HIF-1a accumulation in aconcentration-dependent manner (Fig. 2).

In conclusion, CKA/HP-β-CD complex was found to be the potent inhibitor of hypoxia-induced HIF-1α and to have a strong effect on tumor metastasis. As CKA/HP-β-CD complex had a melanoma selectivity, it is promising for melanoma BNCT agent and inhibitor on spontaneous metastasis of melanoma.

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採択課題番号 25P4-6　メラノーマ中性子捕捉療法への適応を目指した薬物送達システムに関する研究　　プロジェクト

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PR4-6 Design of Boron-Modified Tirapazamine Analogue 1

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INTRODUCTION: Tirapazamine is a potent hypoxic cytotoxin targeting for hypoxic region of solid tumors and showed a radiosensitizing effect [1]. Unfortunately, a phase III trial of tirapazamine found no evidence that the addition of tirapazamine to chemoradiotherapy, in patients with advanced head and neck cancer, improved overall survival [2]. However, if tirapazamine is labeled by boron, reinforcement of the antitumor activity by combined use with a neutron irradiation is expectable. Therefore, we tried a design, synthesis and evaluation of boron-modified tirapazamine analogue.

EXPERIMENTS: The boron-modified tirapazamine analogue **1** (it contains boron isotopes, B-10 and B-11 with their natural abundance ratio) were designed by TX-2137 as a lead compound (Fig. 1). Compound **1** was synthesized at five steps by using 2-nitroaniline as a starting material. *In vitro* antitumor activity was evaluated by WST-1 assay using B16-F10, LLC, KKLS, MKN-45, 293T, and HT-1080 tumor cells. Inhibitory activity for MMP-9 production was evaluated by zymography assay using HT-1080 tumor cells. *In vivo* antimetastatic activity was evaluated by chick embryo model using B16-F10 tumor cells. *In vitro* antitumor activity combined with neutron irradiation by International Electrostatic Confinement Fusion (IECF) was evaluated by WST-1 assay using MCF-7 tumor cells.

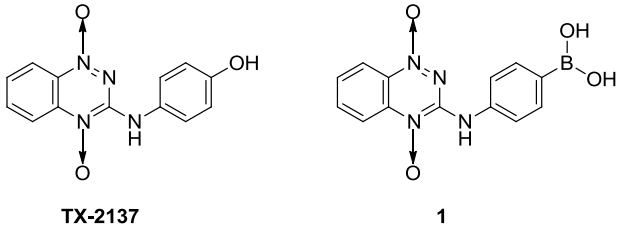

Fig. 1. Chemical structure of TX-2137 and compound **1**.

RESULTS & DISCUSSION: *In vitro* antitumor activity of the compound **1** (49.5 - >100 μM) was very low compared with TX-2137 (1.7 – 10.1 μM) (Table 1). TX-2137 induced apoptosis to MKN-45 tumor cell, although the compound **1** was not it. Inhibitory activity for MMP-9 production of the compound **1** was not inhibited to MMP-9 production, unlike TX-2137 (Fig. 2). Moreover, the compound **1** was not suppressed to metastasis of B16-F10 tumor cells compared with Adriamycin (ADM) and TX-2137 by chick embryo model (Table 2). Combined use of the compound **1** and neutron was not showed a significant sensitizing effect (Table 3). We speculated the reason of low antitumor activity of the compound **1** that the high water-solubility of boric acid to reduce cell permeability of the compound **1**. In conclusion, our designed boron-modified tirapazamine analogue **1** was not candidate for novel hypoxia targeting BNCT drug.

Table 1. *In vitro* antitumor activity of TX-2137 and compound **1**.

	B16-F10	LLC	KKLS
TX-2137 (μM)	4.7	8.7	4.3
1 (μM)	49.5	>100	68.3
	293T	HT-1080	MKN-45
TX-2137 (μM)	1.7	7.9	10.1
1 (μM)	71.7	>100	>100

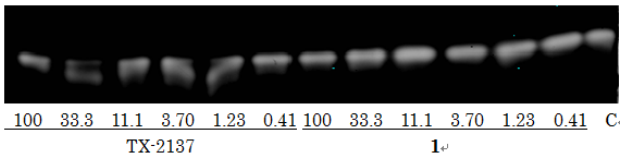

Fig. 2. Inhibitory activity for MMP-9 production of TX-2137 and compound **1** (μM).

Table 2. *In vivo* antimetastatic activity of TX-2137 and compound **1**.

	Adriamycin (40 μg)	TX-2137 (62.5 μg)	**1** (250 μg)
Inhibition ratio (%)	80.6	63.9	0.7
p	0.012	0.029	0.4829

Table 3. Sensitizing activity of compound **1** with neutron.

	Control SF	**1** (100 μM)
Unirradiation	1.00±0.023	1.062±0.005
Neutron (60 min)	1.00±0.053	1.075±0.037

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採択課題番号 25P4-7　ホウ素を有する抗癌剤・分子標的薬剤の分子設計・合成　プロジェクトと機能評価
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PR4-7 The Effect of Intermittent Hypoxia on the Cellular Sensitivities to X-Irradiation and to Boron Neutron Capture Reaction in Rat Glioma Cells

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INTRODUCTION: Recently, intermittent hypoxia whose frequency can range between a few cycles per minutes to hours in tumor is receiving increased attention because of its contribution to tumor malignancy *via* the up-regulation of pro-survival pathways [1]. In regard to the effect of intermittent hypoxia on tumor radiosensitivity, there has been no study to compare the sensitivities to different linear energy transfer (LET) radiations. It is well known that low energy heavy particles (α and Li), produced from boron neutron capture reaction, induces high relative biological effectiveness (RBE). In this study, we aimed to investigate the effect of the preconditioning of intermittent hypoxia on the reproductive cell death induced by X-rays or by boron neutron capture therapy (BNCT) in rat glioma C6 cells.

EXPERIMENTS: C6 cells were grown in D-MEM supplemented with 10% fetal bovine serum under humidified air with 5% CO_2 at 37°C, respectively. Two million cells attached to a 60-mm plastic dish were pretreated with chronic hypoxia (CH) for 4 hours or intermittent hypoxia (IH) with 4 cycles of 1-hour hypoxia interrupted by 30-minute reoxygenation. Hypoxic induction was performed by placing dishes in the commercial hypoxic chamber (MIC-101; Billups-Rothenberg Inc.) and continuously passing a gas mixture of 95% N_2 and 5% CO_2. Concomitant with hypoxic treatment, 10 μg/ml ^{10}B-para-boronophenylalanine (BPA) was incorporated to cells for 4 hours prior to neutron irradiations. Cell suspensions in polypropylene tubes (2×10^6 cells/tube) were irradiated under normoxia either by X-rays from the Shimadzu PANTAK HF-350 X-ray generator or by neutrons from the 1 MW Research Reactor at the KURRI. The total neutron fluencies were measured by gold foil activation analysis. The gamma-ray dose including secondary gamma rays was measured with a Mg_2SiO_4 (Tb) thermo luminescence dosimeter. The calculation of total absorbed dose was performed according to the previous report [2]. Irradiated cells were rinsed, counted, and then diluted to plate onto triplicate a 60-mm plastic dish. After 7 days' incubation, colonies were rinsed with PBS, fixed by 10% formalin solution and stained by 1% methylene blue. The number of colonies consisting of more than 50 cells was counted. The surviving fraction at each dose was calculated with respect to the plating efficiency of the non-irradiated control and dose-response curves were plotted.

RESULTS: Figure 1 shows the survival curves for X-rays and for BNCT obtained from clonogenic assay. In X-irradiated groups, the decrease of cellular radiosensitivity is clearly observed in the IH-treated cells compared to CH-treated cells (Fig. 1A). Reduction ratio of radiosensitivity, which is calculated from the D10 values ($=D10_{IH}/D10_{CH}$), is 1.11. On the other hand, neutron irradiation with BPA suppressed tumor clonogenic abilities largely in all groups, indicating high RBE (Fig. 1B). Note that IH treatment did not decrease radiosensitivity but rather increased it; the reduction ratio is 0.83. These results suggest that BNCT has a good potential to abrogate the IH-induced radioresistance in solid tumor.

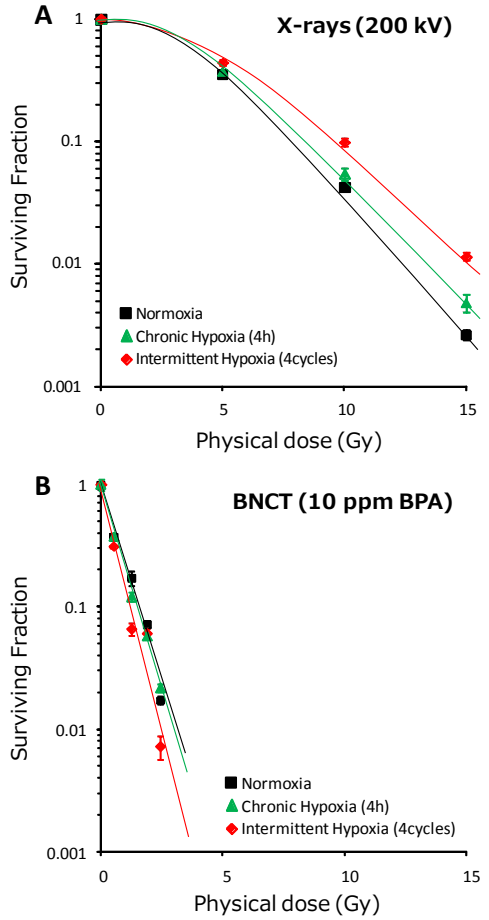

Fig.1 Dose-response curves for C6 cells exposed to X-rays (A) and neutron in the presence of BPA (B).

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採択課題番号 25P4-8　　中性子捕捉療法における間欠的低酸素の生物学的作用に関する基礎的検討　　プロジェクト

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PR4-8 Response of Tumor Cells to BNCT

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INTRODUCTION: Cancer cells usually possess high levels of heterogenieties. The presence of hypoxic cells are known to affect the consequence of the boron neutron capture therapy (BNCT) [1]. Understanding of the molecular mechanism involved in the boron neutron capture reaction (BNCR) might help to know the affecting factors for BNCT. BNCR causes DNA stand breaks and this could lead to cell death processes and cell growth suppression [2]. On the other hand, other cellular responses should be occurred in the cells and these responses may affect cell death and other cellular responses and consequences of BNCT [3]. To carry out comprehensive analysis of cancer cells by transcriptome, proteome, we have carried out setting up experiments in the KUR facility.

EXPERIMENTS: We used human oral squamous cancer SAS cells [1] to perform the comprehensive analysis with transcriptome and proteome analysis. Other cancer cells, human melanoma A375 and SK-MEL5, human pancreatic cancer MIA PaCa2, human glioma U251, human lung cancer A549 were used in parallel for the study of radiation response. The cells were incubated in the polypropyrene vials with or the absence of boronophenylalanine (BPA) (+) and BPA(-) conditions at the concentration of 25 ppm of ^{10}B for 2 hrs. Either 1 MW or 5 MW radiation condition were used. After irradiation in the KUR facility, cells were inoculated for colony formation, RNA and protein preparation or for the immunostaining of gamma-H2AX foci observation. For RNA preparation, Isogen (Dojindo) was used. For protein purification, either Isogen or Laemmli's buffer was mainly used. Proteome analysis was performed using two dimensional-polyacrylamide gel electrophoresis and mass spectrometry. The boron concentration in the culture was measured by prompt-gamma ray analysis with E3 facility. Gold foils were used for monitoring the neutron beam irradiation and TLD detector was also used for the measurement of gamma-irradiation. These physical radiation doses were measured with the kind help of Drs. Yoshinori Sakurai and Hiroki Tanaka of KUR. To analyze the early response of cancer cells, RNA and protein were prepared 6 and 24 hrs after irradiation at the doses of 4 Gy-eq and 24 Gy-eq conditions. Microarray analysis of mRNA in SAS cells was carried out in the National Cancer Center Research Institute in a collaboration with Dr. Yasuhito Arai.

RESULTS:
With colony formation analysis, human oral squamous cancer SAS, human melanoma A375 and SK-MEL5, human pancreatic cancer MIA PaCa2, human glioma U251, human lung cancer A549 showed cell lethal effect in the BNCR condition.
We confirmed the appearance of gamma H2AX foci in BPA (+) condition in SAS, A549 and MIA PaCa2 cells after neutron-beam irradiation.
Genes related to cell death, transcriptional regulation, and inflammatory and immune responses were augmented after BNCR including activating transcription factor (ATF3), early growth response 1 (EGR1), colony stimulating factor 2 (CSF2), interleukin-6, and interleukin-8. By proteome analysis, we found that proteins involved in RNA processing, transcription, DNA repair, and immune response, and endoplasmic reticulum function were found to be increased in the cells. Secretary proteins from BNCR were detected and being studied with the proteome and ELISA analysis. Taken together, it is suggested that diverse irradiation responses could be induced after BNCR reaction as an early response.

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採択課題番号 25P4-9　　BNCT に対する悪性腫瘍の応答性の解析　　プロジェクト
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