VIII-Ⅱ-1. Project Research

Project 6

PR6 Analyzing Tumor Microenvironment and Exploiting its Characteristics for Controlling Malignant Solid Tumors and Distant Metastatic Potential

S. Masunaga

Research Reactor Institute, Kyoto University

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 [3]. 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 [4]. 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 [5].

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 (24P6-1): 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 (24P6-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers
(H. Nagasawa, S. Masunaga, K. Okuda, S. Y. Hirayama and N. Tsurue)

ARS-3 (24P6-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level
(H. Harada, M. Hiraoka, S. Masunaga, S. Itasaka, M. Ogura and M. Yoshimura)

ARS-4 (24P6-4): Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction
(R. Hirayama, S. Masunaga, Y. Sakurai, H. Tanaka and Y. Matsumoto)

ARS-5 (24P6-5): 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)

ARS-6 (24P6-6): 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, K. Li, U. Shu, Y. Kagoshima, K. Hayashi, R. Kawasaki, Y. Hattori and N. Kadono)

ARS-7 (24P6-7): Molecular design and synthesis and functional evaluation of anticancer and molecular targetting agents
(Y. Uto, R. Tada, S, Masunaga)

ARS-8 (24P6-8): Analyzing biological effect of BNCT from the viewpoint of the changes in oxygenation level.
(H. Yasui, S, Masunaga, O. Inanami, T. Yamamori, D. Yamane)

(Underline: Representative at each research group)

REFERENCES:

[1] P. Vaupel, Semin. Radiat. Oncol. **14** (2004) 197-275.
[2] S. Masunaga *et al*., Int. J. Radiat. Oncol. Biol. Phys. **41** (1998) 1163-1170.
[3] S. Masunaga *et al*., J. Cancer Res. Clin. Oncol. **125** (1999) 609-614.
[4] S. Masunaga *et al*., Int. J. Radiat. Oncol. Biol. Phys. **44** (1999) 391-398.
[5] S. Masunaga *et al*., Clin. Exp. Metastasis **26** (2009) 693-700.

採択課題番号 24P6　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がん微小環境解析とその応用　プロジェクト

（京大・原子炉）増永慎一郎

PR6-1 Combined Treatment with Continuous Administration of Tirapazamine and Mild Temperature Hyperthermia in γ-Ray Irradiation in Terms of Local Tumor Response and Lung Metastatic Potential

S. Masunaga, Y. Sakurai, H. Tanaka, K. Tano, M. Suzuki, N. Kondo, M. Narabayashi, A. Maruhashi and K. Ono

Research Reactor Institute, Kyoto University

BACKGROUNDS AND PURPOSES: Many of the cancer cells in solid tumors are non-proliferating (quiescent), and many of the features of quiescent (Q) cells are still unknown. To improve cancer treatment, the response of Q cells to anticancer treatment should be determined, since often tumor cells that are quiescent *in situ* are still clonogenic.

The development of bioreductive agents that are particularly toxic to hypoxic cells is considered a promising approach to solving the problem of radio-resistant tumor hypoxia in cancer radiotherapy. Tirapazamine (TPZ), a lead compound in the development of bioreductive hypoxic cytotoxins, in combination with radiation has been shown to be very useful for controlling solid tumors as a whole, especially for controlling Q tumor cell populations that are rich in hypoxic region. Tumor hypoxia results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia, transient hypoxia or ischemic hypoxia). Chronically hypoxic tumor cells existing at the rim of the oxygen diffusion distance can be killed by just a single administration of TPZ. Acutely hypoxic tumor cells occurring sporadically throughout solid tumors can be killed by TPZ during long-term continuous administration. Namely, the long-term continuous administration of TPZ can kill both chronically and acutely hypoxic tumor cells.

Mild temperature hyperthermia (MTH) was reported to increase the response of tumors to radiation by improving oxygenation through an increase in tumor blood flow. Further, MTH was also shown to enhance the tumor response, especially of the intratumor Q cell population, to TPZ.

Metastasis is a leading cause of cancer deaths and involves a complex, multistep process by which tumor cells disseminate to distant sites to establish discontinuous secondary colonies. It was reported that acute and cyclic, but not chronic, hypoxia significantly increased the number of spontaneous lung metastases in mice by a factor of about 2, and that this effect was due to the influence of the acute hypoxia treatment on the primary tumor and not to other potential effects of the treatment such as damage to the lung epithelium. Based on this report, we recently reported the significance of injections of an acute hypoxia-releasing agent, nicotinamide, into tumor-bearing mice as a combined treatment with high dose rate γ-ray irradiation in terms of reducing the number of lung metastatic nodules.

Here, using a readily metastasizing murine melanoma cell line, we tried to analyze the usefulness of combined treatment with continuous long-term administration of TPZ and MTH in radiotherapy with γ-rays in terms of local tumor response and lung metastatic potential. Concerning the local tumor response, the effect not only on the total (= proliferating (P) + 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 [1].

MATERIALS AND METHODS: B16-BL6 melanoma tumor-bearing C57BL/6 mice were continuously given 5-bromo-2'-deoxyuridine (BrdU) to label all proliferating (P) cells. The tumor-bearing mice then received γ-ray irradiation after a single intraperitoneal injection or 24 h continuous subcutaneous infusion of TPZ, either with or without MTH. Immediately after the irradiation, cells from some tumors were isolated and incubated with a cytokinesis blocker. The responses of the Q and total (= P + Q) cell populations were assessed based on the frequency of micronuclei using immunofluorescence staining for BrdU. In other tumor-bearing mice, 17 days after irradiation, macroscopic lung metastases were enumerated.

RESULTS: Continuous administration elevated the sensitivity of both the total and Q cells, especially the total cells. MTH raised the sensitivity of Q cells more remarkably in both single and continuous administrations, probably because of more exposure to TPZ in intermediately hypoxic areas derived mainly from chronic hypoxia through MTH. With or without irradiation, TPZ, especially administered continuously and combined with MTH, decreased the number of lung metastases.

DISSCUSSION: The combination of continuous long-term administration of TPZ and MTH in γ-ray irradiation was thought to be promising because of its potential to enhance local tumor response and repress lung metastatic potential [2].

It was elucidated that control of the chronic hypoxia-rich Q cell population in primary solid tumors has the potential to impact the control of local tumors as a whole, while control of the acute hypoxia-rich total tumor cell population has the potential to impact the control of lung metastases. Namely, in conventional radiotherapy, continuous TPZ administration combined with MTH is thought to have a great potential to control both local solid tumors and lung metastases from the local tumors.

REFERENCES:

[1] S. Masunaga *et al.*, J. Radiat. Res. **43** (2002) 11-25.

[2] S. Masunaga, *et al.*, Int. J Hyperthermia **28** (2012) 636-644.

採択課題番号 24P6-1　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

（京大・原子炉）増永慎一郎、櫻井良憲、田中裕基（藍野学院短期大）高垣政雄（福井大）松本英樹（済生会千里病院）山本直典（大阪医大）弘田祐己

PR6-2 Development of Direct Intra-Cellular Drug Delivery System Based on Lipidated Peptides, Pepducins

M. Tsuji, S. Masunaga[1], K. Okuda, T. Hirayama and H. Nagasawa

Laboratory of Medicinal & Pharmaceutical Chemistry, Gifu Pharmaceutical University
[1] *Research Reactor Institute, Kyoto University*

INTRODUCTION: One of the most important issues in the development of the boron carrier for boron neutron capture therapy (BNCT) is the innovation of versatile drug delivery systems across the cell membrane. We focus on the unique cell-penetrating feature of lipidated peptides called pepducins discovered as allosteric modulators of GPCRs[1]. Unlike classical GPCR modulators that act at the extracellular ligand-binding site of receptors without crossing the plasma membrane barrier, pepducins are supposed to interact with the intracellular portion of target receptors on the inner leaflet of the lipid bilayer. Pepducins consist of a synthetic peptide derived from the appropriate intracellular loop domains of GPCRs, typically 10–20 amino acids in length, and a hydrophobic moiety, e.g., palmitate. The lipid moiety anchors in the plasma membrane and sequentially flips across the membrane. Then, pepducins directly penetrate cellular membrane not by endocytosis to interact with the intracellular loop domain of the GPCR. We envisage that the transmembrane system of pepducin could be useful for the development of the cell penetrating boron carrier.

We report the design and synthesis of novel bioreductively activatable FRET probes to analyze the transbilayer movement of lipidated peptides in living cells by fluorescence microscopy. We further demonstrate substantial functions of the lipidated peptide moiety to flip across the membrane and to achieve rapid delivery of fluorescein as a cargo into the cytosol using pepducin-based probes [2].

EXPERIMENTS: To visualize the flipping movement of pepducins across the membrane in live cells, we designed two types of FRET-based imaging agents, Pep13-FL-SS-Dab (**1**) and Pep13-Dab-SS-FL (**2**), which were based on the structure of the PAR1 pepducin agonist, P1pal-13, developed by Covic *et al.*[1]. he probes are composed of pepducin and a FRET component consisting of 5(6)-FAM as fluorophore and dabcyl as quencher, connected through disulfide bond linkage (Figure 1). All the peptide parts of the probes were obtained by Fmoc solid-phase synthesis (SPPS). We performed live cell imaging experiments using MCF-7 cells with probe **1** and **2** and confocal microscopy. MCF-7 cells were treated with the probes at the concentration of 1 μM for 15 min, and then observed with the confocal laser-scanning microscope after changing the medium and washing. Nuclear co-staining with Hoechst 33342 (10 mg/mL) was also demonstrated before the treatment with the probe.

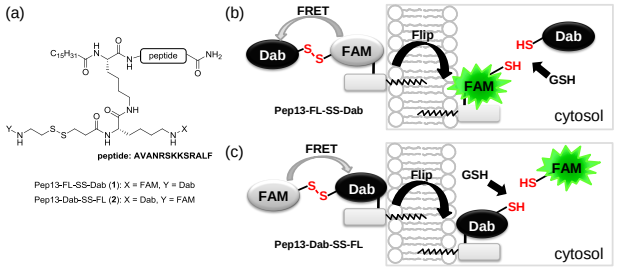


Fig. 1 Design and synthesis of bioreductively activatable FRET based probes.

RESULTS: Probe **1**-treated cells showed a distinct membrane- localized fluorescence pattern (Figure 2a). While, probe **2**-treated cells showed diffuse cytosolic fluorescence (Figure 2b). When the cells were pre-incubated with *N*-ethyl- maleimide (NEM) before the treatment with probe **1** in order to deplete reduced thiols both in medium and in the cells, fluorescence signals were significantly low (Figure 3c), indicating that the fluorescence enhancement is definitely attributed to free thiols.

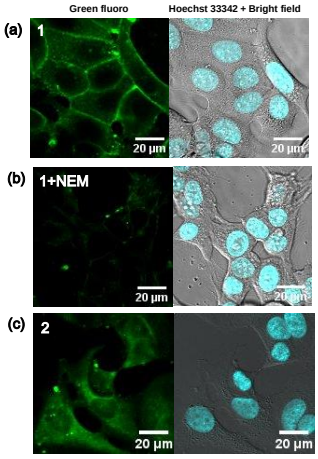


Fig 2. Confocal microscopic images and bright field (BF) images of live MCF-7 cells. (a) 1 μM of Pep13-FL-SS-Dab (**1**) for 15 min . (b) 1 μM of Pep13-Dab-SS-FL (**2**) for 15 min. (c) Cells were incubated with 50 μM of NEM prior to treatment with 1 μM of **1** for 15 min.

CONCLUSION: We developed novel bioreductively activatable FRET probes based on pepducins. These probes enabled for the first time live cell imaging of the unique transbilayer movement of pepducins, which revealed that the lipid moiety of pepducin plays a pivotal role to flip across the plasma membrane and to tether its peptide moiety to the inner leaflet of the bilayer. Furthermore, these studies demonstrated the great potential of applying the present probe architect to simple and straightforward intracellular drug delivery systems. Currently, we are optimizing the peptide sequence and lipid structure for intracellular targeting of different cells for the development of pepducin-based drug delivery systems.

REFERENCES:

[1] L. Covic *et al.*, Proc. Natl. Acad. Sci. USA., **99** (2002) 643–648.
[2] M. Tsuji *et al.*, Org. Biomol. Chem., **11 (**2013), 3030-3037.

採択課題番号 24P6-2　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
（岐阜薬大・薬化学）辻美恵子、鶴江範彦、奥田健介、平山　祐、永澤秀子（京大・原子炉）増永慎一郎

PR6-3 Upstream Activators of HIF-1 as Targets for Radiosensitization

H. Harada, S. Masunaga[1] and M. Hiraoka[2]

Group of Radiation and Tumor Biology, Career-Path Promotion Unit for Young Life Scientists, Kyoto University.
[1]Research Reactor Institute, Kyoto University
[2]Department of Radiation Oncology and Image-applied Therapy, Graduate School of Medicine, Kyoto University.

INTRODUCTION: Effect of conventional therapies for cancer patients, such as chemotherapy, radiation therapy, and surgery, is known to be affected by various intracellular and extracellular factors in malignant tumors. One of the most influential factors is activity of a transcriptional factor, hypoxia-inducible factor 1 (HIF-1). 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 transactivating activity of HIF-1α, which are strictly regulated by α-KG-dependent enzymes, prolyl-4-hydroxylases (PHDs) and factor-inhibiting HIF-1 (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 the improvement of oxygen-availability (angiogenesis), adaptation of cellular metabolism to hypoxia (metabolic reprogramming), and escape from hypoxia (invasion and metastasis of cancer cells). Indeed, clinical as well as basic researches have showed that HIF-1α expression level correlates with a poor prognosis and incidences of both tumor recurrence and distant tumor metastasis, justifying targeting HIF-1 and its upstream activators for cancer therapy. However, molecular mechanisms behind the activation of HIF-1 have not been fully elucidated yet, which makes it difficult to develop strategies for eradication of cancers.

EXPERIMENTS & RESULTS: In order to explore novel genes which are responsible for the activation of HIF-1, we established a new screening method (Fig. 1). Using an artificial promoter composed of five repeats of hypoxia-response elements (HRE) and human CMV minimal promoter (5HRE promoter: 5HREp), we newly constructed a plasmid expressing blasticidin S resistant gene (*bsd*) in a HIF-1-dependent manner (*5HREp-bsd* herein). NIH3T3 cells stably transfected with the *5HREp-bsd* (NIH3T3/5HREp-bsd) were sensitive to blasticidin S under normoxic conditions because of insufficient expressions of both HIF-1α and BSD. After introducing human cDNA library into the NIH3T3/5HREp-bsd cells, we cultured them in blasticidin S-containing medium under normoxic conditions with an expectation that some cDNAs encode upstream activators of HIF-1 and consequently induce BSD expression.

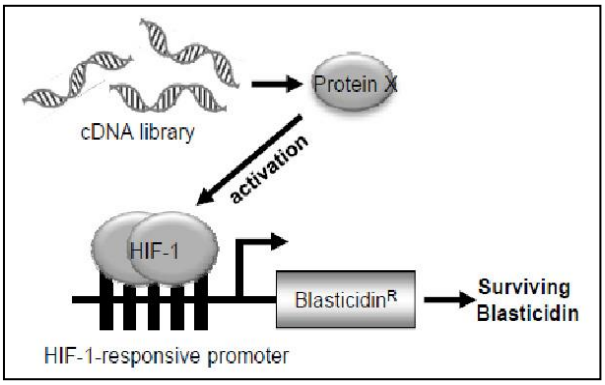


Fig. 1. Schematic Diagram of the Gene Screening Method for Novel Activators of HIF-1.

Through such a gene screening experiment, we could actually acquire surviving colonies which showed blasticidin S-resistance compared to the parental cell line. We rescued cDNA from one of the colonies by PCR and identified it as the gene encoding wild type isocitrate dehydrogenase 3alpha (IDH3α), a subunit of IDH3 composed of two α, one β, and one γ subunits. In order to examine whether IDH3α induces HIF-1 activity, we performed a luciferase assay using the *5HREp-luc* reporter gene, which expresses Luciferase under the control of 5HREp. Overexpression of IDH3α resulted in up-regulation of HIF-1 activity in various cancer cell lines under normoxic and hypoxic conditions. We plan to examine whether targeting IDH3α enhances therapeutic effect of radiation in next experiments [1-3].

REFERENCES (papers published from our lab):

[1] Zhu Y *et al*., Oncogene, **32** (2013) 2058-2068.
[2] Yoshimura M *et al*., Biomed Res Intl., (2013) Article ID 685308.
[3] Yeom CJ *et al*., Int J Mol Sci. **13** (2012) 13949-13965..

採択課題番号 24P6-3　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機能解析、および局所腫瘍制御への展開　プロジェクト

（京大・キャリアパス）原田浩（京大・原子炉）増永慎一郎（京大院・医）平岡真寛

PR6-4 BPA Concentration-Dependency of HSG Cell Inactivation by Neutron Irradiation

R. Hirayama, Y. Matsumoto, S. Masunaga[1], Y. Sakurai[1], H. Tanaka[1] and Y. Furusawa

Research Center for Charged Particle Therapy, National Institute of Radiological Sciences
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: Excellent dose distribution of neutron capture reaction of boron atom induces high relative biological effectiveness (RBE) and the low oxygen enhancement ratio (OER). These phenomena are commonly assumed to be an interaction between cells and low energy heavy particles (α and Li) resulting from the boron atom fissions in the cells. However, there has been little study done concerning the action of the particles on living cells. We have investigated contributions of indirect actions of radiation in cell killing by heavy ions with radical scavenger that selectively reduces the indirect action [1-3].

Therefore, it is important that how these mechanisms can be made to clear through a thorough basic research in boron neutron capture therapy is urgently discussed. The main object of this year is to make clear an influence of boron neutron capture reaction at high concentration of ^{10}B-para-boronophenylalanine (BPA) on HSG cell inactivation.

EXPERIMENTS: Human salivary gland tumor (HSG) cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under humidified air with 5% CO_2 at 37˚C. The HSG cells were incubated with 25 μg/ml BPA for 3 h before irradiation. The cells were suspended at a density of about 7×10^5 cells/ml containing 25 μg/ml BPA. The cells in Polypropylene tubes (NUNC) were irradiated at the remodeled heavy water facility at the KURRI.

Total fluencies of thermal neutron, epithermal neutron and fast neutron were measured by means of gold foil activation analysis. The gamma ray dose including secondary gamma rays was measured with a thermo luminescence dosimeter. Boron concentrations in the cells were taken to be equivalent to those in the medium as reported previously [4].

After irradiation, cells were seeded in triplicate onto 60 mm (Φ) culture dishes at densities to give approximately 100 colonies per dish. After 14 days of incubation, the colonies were fixed with 10 % formalin solution and stained with 1 % methylene blue in water. Colonies consisting of more than 50 surviving cells were scored. The survival curves were fitted by the single-hit model: $SF = \exp(-\alpha \cdot D)$. SF and D are the surviving fraction and the dose (Gy^{-1}), respectively.

RESULTS: The survival curves for neutrons are shown in Fig. 1. The D_{10} value of neutron with and without BPA were 0.2 and 2.8, respectively. The D_{10} value of X-rays was 4.7 Gy. The RBE values for with and without BPA were 19.9 and 1.7, respectively. The survival curves of the coefficient of determination (R^2) were above 0.8. We will retest and add the dose points and BPA concentrations. These results suggest that he large RBE was induced by low energy heavy particles (α and Li) from boron neutron capture reaction.

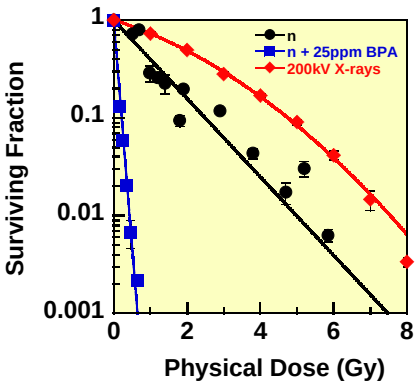


Fig. 1. Survival curves for HSG cells exposed to neutrons in the absence (circle) and presence (square) of 25 μg/ml BPA. The survival parameters were calculated from the data by a curve fitting using: $SF = \exp(-\alpha \cdot D)$.

REFERENCES:

[1] A. Ito *et al.*, Radiat. Res., **165** (2006) 703-712.
[2] R. Hirayama *et al.*, Radiat. Res., **171** (2009) 212-218.
[3] R. Hirayama *et al.*, Radiat. Phys. Chem., **78** (2009) 1175-1178.
[4] Y. Sakurai and T. kobayashi, Nucl. Instrum. Methods Phys. Res. Sect. A., **453** (2000) 569-596.

採択課題番号 24P6-4　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（放医研・重粒子医科学センター）平山亮一、松本孔貴、古澤佳也（京大・原子炉）増永慎一郎、櫻井良憲、田中浩基

PR6-5 Electroendocytosis of Boron-Conjugated Hyaluronic Acid NanoParticles for Tumor Targeting in Boron Neutron Capture Therapy

S. Kasaoka, K. Hashimoto, S. Masunaga[1], Y. Sakurai[1], H. Tanaka[1] and K. Ono[1]

Department of Pharmaceutical Science, Hiroshima International University
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: Hyaluronic acid (HA) is a candidate for active targeting ligand to melanoma, many of which overexpress the hyaluronic acid receptors CD44 and RHAMM [1]. Electroendosytosis, low electric fields (LEF) induced endocytic-like process, is novel concept as a complementary method to uptake of macromolecules by cells [2]. Such uptake is induced by the production of hydrogen ions with electrolysis [3]. The elevated concentration of extracellular hydrogen ions which bind to the anionic charged sites on the external membrane leaflet would result in local reduction of charge density on the external membrane surface. In this study, we have developed a novel boron delivery system using the combination of the electroendosytosis and HA conjugated with borocaptate (BSH) for boron neutron capture therapy (BNCT). This BSH-bearing HA nanoparticles (B-HA-NPs) are expected to deliver boron atoms into melanoma cells by electoroendosytosis and receptor-mediated endocytosis for antitumor application in BNCT.

EXPERIMENTS: BSH was dissolved in acetone and added to N-Succinimidyl 6-Maleimidobenzoate with triethylamine catalysis. The mixture was incubated at room temperature for 2 h under argon. Sodium hyaluronate (M.W. = 4,200, 78,000, 960,000) was dissolved in deionized water. To this solution, the highly acidic ion exchange resin, Dowex-100, was added, and the slurry was stirred for eight hours. The acidic solution was neutralized with 0.2M tetrabutylammonium hydroxide, forming a quaternary ammonium salt of hyaluronate and the tetrabutylammonium group (HA-TBA). The solution was frozen and lyophilized to yield the dry product. The HA-TBA was dissolved in DMSO and added to BSH derivative was of conjugation with diethylamine catalysis. The mixture was incubated at room temperature for 48 h under argon. The solution was dialyzed and lyophilized to yield the dry product.

B16F10 murine melanoma cells were pre-incubated with 25 ppm of B-HA-NPs at 37°C for 2 hours before neutron irradiation. The exposure of cells to pulsed LEF was performed in 24 well plate between parallel stainless steel electrodes (20 V/cm). 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: B-HA-NPs had high stability (89-95%) in the retention of ^{10}B during storage at 4-37°C for 2 weeks. All borocaptate-loaded formulations had no cytotoxic effects. B-HA-NPs (M.W.=78,000, 960,000) were readily bound to melanoma cells, and were internalized by electroendocytosis and receptor-mediated endocytosis. As shown in Fig. 1, B-HA-NPs with exposure to pulsed LEF showed higher suppression of growth of melanoma cells than without exposure to pulsed LEF. This result suggested novel boron delivery system using the combination of the electroendocytosis and receptor-mediated endocytosis of B-HA-NPs is useful in BNCT.

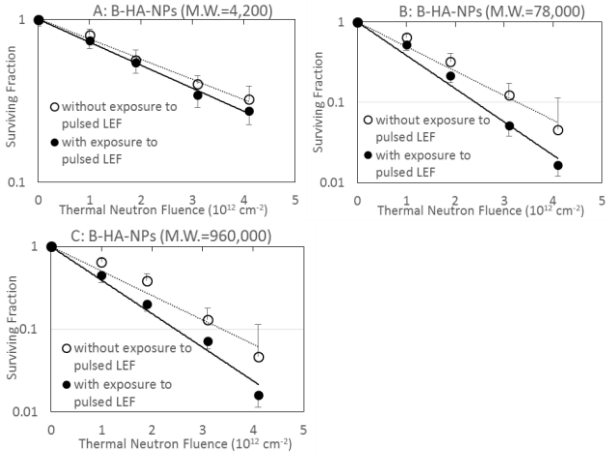


Fig. 1. Suppression of the colony formation of B16F10 cells after in vitro BNCT.

REFERENCES:

[1] D. Peer and R. Magalit, Neoplasia, **6** (2004) 343-353.
[2] Y. Antov *et al*., Biophy J., **88** (2005) 2206-2223.
[3] N. Ben-Dov *et al*., PLoS One, **7** (2012) 50299-50306.

採択課題番号 24P6-5　新規皮膚透過性メラノーマ標的化ボロン送達システムを用いた中性子捕捉療法の開発　プロジェクト

（広国大・薬）笠岡　敏、橋本佳奈（京大・原子炉）増永慎一郎

PR6-6 Kojic Acid-Appended Carorane/Hydroxypropyl-β-Cyclodextrin Complex for BNCT of Murine Melanoma

R. Kawasaki, T. Nagasaki, J. Li, H. Azuma, S. Masunaga[1], Y. Sakurai[1], M. Kirihata[2] and Y. Hattori[2]

Graduate School of Engineering, Osaka City University
[1]Research Reactor Institute, Kyoto University
[2]Graduate School of Life and Environmental Sciences, Osaka Prefecture University

INTRODUCTION: Boron neutron capture therapy (BNCT) has been attracted great deal of attention as a potentially useful modality for malignant melanoma [1]. The selective and effective delivery of ^{10}B compounds into melanoma cells is a key process in order to improve the efficacy of melanoma BNCT. Kojic acid is well known to work as an excellent whitening agent for melanocytes by a strong tyrosinase inhibition [2]. This fact suggests that kojic acid possess a specific affinity for melanocytes. Previously, we evaluated kojic acid appended o-carborane (CKA) as BNCT boron agent for melanoma. Kojic moiety functionalized not only to accumulate selectively into melanoma cells, but also to localize nuclei. As these results indicated that CKA was promising to be a boron agent for melanoma BNCT, herein, we evaluated CKA as BNCT agent *in vivo*.

EXPERIMENTS: Water-soluble complex of CKA with hydroxypropyl-β-cyclodextrin (CKA/HP-β-CD, Fig. 1) was prepared with high-speed vibration milling technique [3]. B16BL6 murine melanoma cells were grown in RPMI mediums containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C with CO_2 atmosphere. C57/BL6J mice were transplanted with 2 x 10^5 B16BL6 cells into the left thigh. After 10 days of transplantation, intraperitoneal injections of boron compounds (1500 ppm or 4500 ppm of ^{10}B concentration; 200 μl) were carried out for the evaluation of tumor accumulation and BNCT effect. Boron concentration was measured by ICP-AES (Vista-MPX, Seiko instruments Inc.).

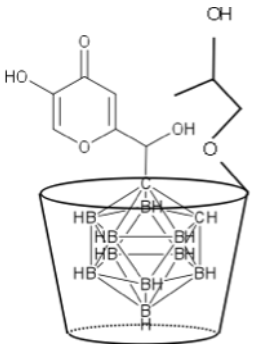


Fig. 1. Water-soluble CKA/HP-β-CD complex.

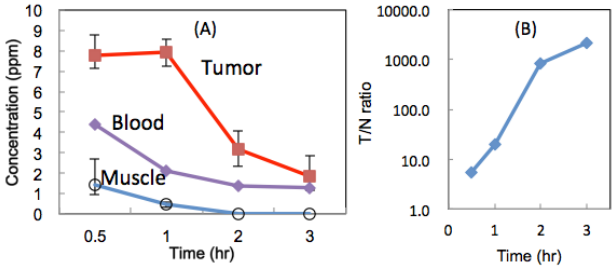


Fig. 2. Tumor-accumulation of boron in tumor-bearing mice using CKA/HPβ-CD (A). The tumor to normal tissue ratio (T/N) (B).

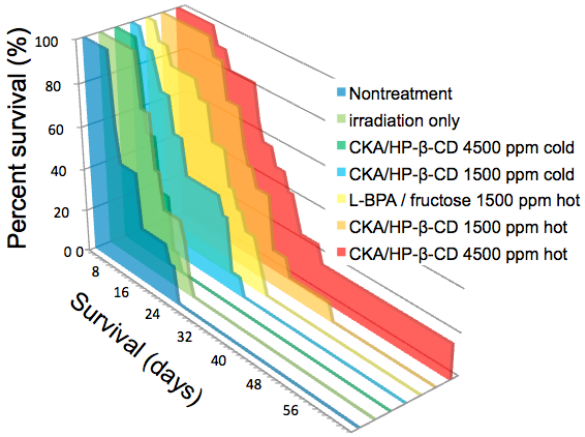


Fig. 3. Survival curves of tumor-bearing mice after neutron irradiation (5.0 x 10^{12} fluence cm^{-2}) in KUR atomic reactor.

RESULTS AND DISCUSSION: *In vivo* tumor-accumulation studies were performed with CKA/HP-β-CD complex (Fig. 2). After 1 hour of administration, highest concentration (ca. 8 ppm of ^{10}B) was observed. These phenomena are consistent with *in vitro* melanoma selectivity. The antitumor effect of BNCT was evaluated on basis of the survival of mice (Fig. 3). All the non-treated mice did not survive after 22 days of neutron irradiation, and their average survival rate was 14 days. When 1500 ppm of ^{10}B solutions were used, average survival rate were 19 and 22 days with L-BPA/fructose and CKA/HP-β-CD complex, respectively. Moreover, long survival rates (25 daays) were observed with 4500 ppm of ^{10}B solutions of CKA/HP-β-CD complex. Kojic acid-appended carborane (CKA) is promising to be an efficient boron agent toward melanoma BNCT.

REFERENCES:

[1] H. Fukuda *et al.*, Radiat. Res., **138** (1994) 435-442.
[2] Y. Mishima, Pigment Cell Res., **14** (2001) 47-70.
[3] K. Komatsu *et al.*, J. Chem. Soc., Perkin Trans. 1, (1999) 2963-2966.

採択課題番号 24P6-6　メラノーマ中性子捕捉療法への適応を目指した薬剤送達システムに関する研究　　プロジェクト

（阪市大・工）長﨑　健、東　秀紀、李　家暐、鹿子嶋祐太、林高一郎、河崎　陸、櫻本昌士、湯川寛子（阪府大・生命環境科）切畑光統、服部能英、門野尚之（京大・原子炉）増永慎一郎

PR6-7　Design, Synthesis and Phrmacokinetic Analysis of Boron Tracedrugs UTX-51

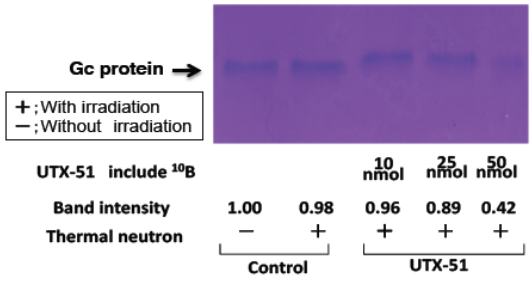
Y. Uto, R. Tada, H. Hori and S. Masunaga[1]

Institute of Technology and Science, Graduate School The University of Tokushima
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: The evaluation of pharmacokinetic properties and ADME-tox of drug candidates, being under development, and drugs, available on the market, has recently become an increasingly important factor of drug discovery and development, because of increased needs of targeted drugs with less adverse effects. This needs accelerate medicinal chemists to develop drugs with higher traceability, even in their whole lifetime. Radiolabeled compounds have been still available with some inherent problems such as their half-life and the specific regulation of experimental facilities. For the purpose of overcoming these problems and creating traceable drugs without RI forever, we are developing boron tracedrugs with their "on demand" traceability and their physical force for neutron dynamic therapy (NDT). We previously developed boron tracedrugs UTX-42, UTX-43, and UTX-44, which possess antioxidant potency [1]. Among boron tracedrugs tested previously, we choose the boron tracedrug, UTX-51, for our present NDT study to explore their dynamic, beyond chemical, effects when acquired by weak thermal neutron irradiation of human serum Gc protein, which is related with serum protein-quality control, treated with the boron tracedrug UTX-51.

EXPERIMENTS: The curcuminoid boron tracedrugs UTX-51 (it contains boron isotopes, B-10 and B-11 with their natural abundance ratio) was used for neutron dynamic therapy. Thermal neutron irradiation was performed using a reactor neutron beam with a cadmium (Cd) ratio of 9.4. The neutron fluence was measured from the radioactivation of gold foils at the front of the sample tubes, and the average neutron fluence determined from the values measured was used. Contaminating γ-ray doses, including secondary γ-rays, were measured with thermoluminescence dosimeter powder at the front of the sample tubes. The absorbed dose was calculated using the flux-to-dose conversion factor. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the decomposition by thermal neutron irradiation of human serum Gc protein treated with the boron tracedrug UTX-51. The evaluation of the pharmacokinetic property of UTX-51 is performed using chick embryo model. After UTX-51 was intravenously injected into blood vessel in the chorioallantoic membrane, each internal organs were isolated and the boron concentration was measured by neutron capture prompt gamma activation analysis (PGAA).

RESULTS: The combination of 0.5 nmol Gc protein (54 KD, 27 μg) with three different B-10 concentration of 10, 25. and 50 nmol of the boron tracedrug UTX-51 (MW 416.12 g/mol; 50 nmol or 20.8 μg, 125 nmol or 52.0 μg, and 250 nmol or 104.0 μg, for 10 nmol B-10, 25 nmol B-10, and 50 nmol B-10, respectively) showed a decrease in band intensity after neutron irradiation (Fig. 1). The pharmacokinetic property of UTX-51 by chick embryo model was shown in Fig. 2. Although the concentration of UTX-51 in the heart and a brain decreased with time progress, while it's rose 12 hours after injection in blood and decreased after that.
In conclusion, all doses of the boron tracedrug UTX-51 caused destructive dynamic damage against Gc protein during thermal neutron irradiation.



GcProtein treated with boron tracedrug on SDS-PAGE gel stained with Coomassie Brilliant Blue (CBB) and Band intensity. Irradiation: thermal neutron (Absorbed dose 0.44 Gy at the present of 10 nmol ^{10}B ; Time 45 min). Band intensity were measured with SWEDAY Just TLC soft (control = 1.00).

Fig. 1. NDT of Gc protein using a boron tracedrug UTX-51.

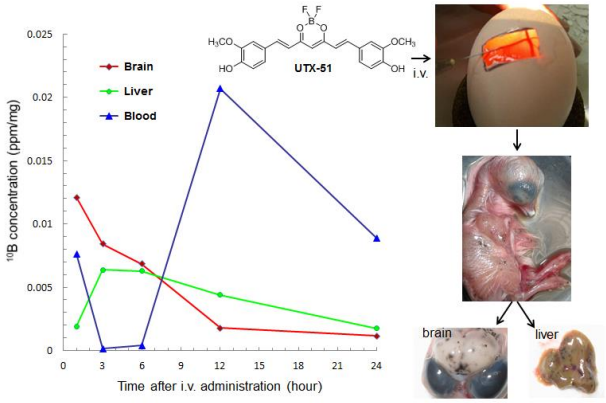


Fig. 2. Pharmacokinetic property of UTX-51 by chick embryo model.

REFERENCES:

[1] E. Nakata *et al.*, Adv. Exp. Med. Biol., **737** (2012) 301-306.

採択課題番号 24P6−7　ホウ素を有する抗癌剤・分子標的薬剤の分子設計・合成と機能評価　プロジェクト
（徳島大・ソシオテクノサイエンス）宇都義浩、多田　竜、堀　均（京大・原子炉）増永慎一郎

PR6-8 The Effect of Boron Neutron Capture Reaction on the Cell Survival of Tumor Cells Preconditioned with Intermittent Hypoxia

H. Yasui, M. Nagane, S. Masunaga[1], T. Yamamori and O. Inanami

Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: Hypoxia has been known to be a feature associated with tumor radioresistance. Recently, in addition to chronic hypoxia due to the limitation of the oxygen diffusion, intermittent hypoxia whose frequency can range between a few cycles per minutes to hours is receiving increased attention [1]. It is because this type of hypoxia has been reported to have an influence on tumor malignancy through the up-regulation of pro-survival pathways [2]. Our previous study revealed that the preconditioning of murine squamous cell carcinoma (SCCVII) cells with intermittent hypoxia decreased their sensitivity to X-irradiation compared to persistent hypoxia or normoxia. However, it has not been investigated whether intermittent hypoxia induces cellular radioresistance to the high-linear energy transfer (LET) radiation as well as the low-LET X-irradiation. 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 reveal the effect of the preconditioning of intermittent hypoxia on the reproductive cell death induced by boron neutron capture reaction in SCCVII cells.

EXPERIMENTS: SCCVII cells were grown in minimum essential medium alpha supplemented with 10% fetal bovine serum under humidified air with 5% CO_2 at 37°C. Two million cells attached to a 60-mm plastic dish were pretreated with persistent hypoxia for 4 hours or intermittent hypoxia with 4 cycles of 1-hour hypoxia interrupted by 30-minute reoxygenation, according to the protocol illustrated in Fig. 1. 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 irradiations. Cell suspensions in polypropylene tubes (2×10^6 cells/tube) were irradiated under normoxia 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 [3]. 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.

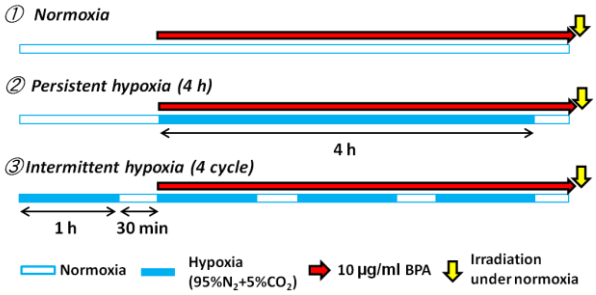


Fig. 1. Schematic experimental protocols.

RESULTS: We show the preliminary result of clonogenic assay in Fig. 2. It is unclear whether the survival curve has an initial linear slope derived from contaminated gamma-ray in each group. The enhancement rate by BNCT obtained from the D10 values (1.75 Gy and 4.00 Gy for BPA[+] and BPA[-], respectively) was about 2.29. There was no difference in the radiosensitivities among the three groups pretreated with normoxia, persistent hypoxia, or intermittent hypoxia. We need to make more detailed investigations to compare with the case of photon beam.

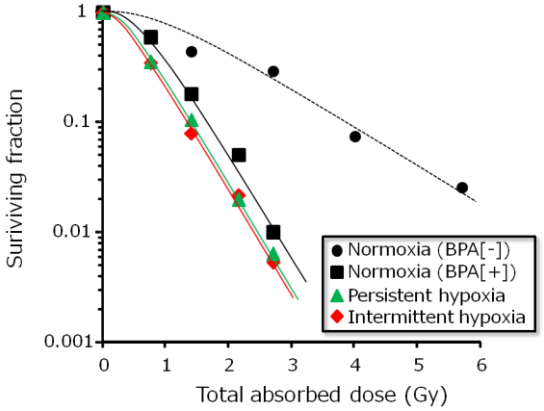


Fig.2. Dose-response curves for SCCVII cells.

REFERENCES:

[1] H. Yasui *et al.*, Cancer Res., **70** (2010) 6427-6436.
[2] M.W. Dewhirst *et al.*, Radiat. Res., **172** (2009) 653-665.
[3] Y. Kinashi *et al.*, Radiat. Oncol., **6** (2011) 106-113.

採択課題番号 24P6-8　　酸素分圧変動に着目した中性子捕捉療法の生物学的効果に関する基礎的検討　　プロジェクト

（北大・獣医）安井博宣、永根大幹、山盛　徹、稲波　修（京都大・原子炉）増永慎一郎