I. Project Research

Project 7

PR7 Analyzing Tumor Microenvironment and Exploiting its Characteristics in Search of Optimizing Cancer Therapy Including Neutron Capture Therapy

S. Masunaga

Research Reactor Institute, Kyoto University

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 or bevacizumab may be promising for reducing numbers of lung metastases. Consequently, BPA-BNCT in combination with nicotinamide and/or bevacizumab treatment 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 (28P7-1): 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 (28P7-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers. (H. Nagasawa, *et al.*)

ARS-3 (28P7-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level. (H. Harada, *et al.*)

ARS-4 (28P7-4)*: Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction. (R. Hirayama, *et al.*)

ARS-5 (28P7-5)*: Transdermal Drug Delivery System using Hyaluronan-Conjugated Liposomes as ^{10}B-Carrier in Boron Neutron Capture Therapy for Melanoma (S. Kasaoka, *et al.*)

ARS-6 28P7-6) *: Evaluation of Inclusion Complex of Carborane Modified Kojic Acid and Cyclodextrin as ^{10}B-Carrier in Boron Neutron Capture Therapy. (T. Nagasaki, *et al.*)

ARS-7 (28P7-7)*: Molecular Design and Synthesis and Functional Evaluation of Anticancer and Molecular Targeting Agents. (Y. Uto, *et al.*)

ARS-8 (28P7-8)*: Analyzing Biological Effect of BNCT from the Viewpoint of the Changes in Oxygenation Level. (H. Yasui, *et al.*)

ARS-9 (28P7-9): Analyses on the Responsiveness of Malignant Tumors to BNCT. (M. Masutani, *et al.*)

ARS-10 (28P7-10) *: Assay for Tumor Cell Survival and Tumor Growth Delay through Neutron Capture Reaction according to the Changes in Intracellular Concentrations within Solid Tumors of Newly- Developed ^{10}B-Carriers. (K. Nakai, *et al.*)

ARS-11 (28P7-11)*: Antitumor and Metastasis-Reppresing Effect of BNCT on Human Breast and Pancreatic Cancer Cell Lines. (Y. Matsumoto, *et al.*)

(*Due to the abscence of operating our reactor in 2016, no data were obtained, resulting in no reporting here.**)**

PR7-1 Effect of Oxygen Pressure during Incubation with a ^{10}B-carrier on ^{10}B Uptake Capacity of Cultured *p53* Wild-type and Mutated Tumor Cells, Referring to Dependency on *p53* Status of Tumor Cells and Types of ^{10}B-Carriers

S. Masunaga, H. Tatebe[1], Y. Nishimura[1], K. Tano, Y. Sanada, Y. Sakurai, H. Tanaka, M. Suzuki, N. Kondo, T. Watanabe, Y. Tamari, T. Takata, A. Maruhashi and K. Ono

Research Reactor Institute, Kyoto University
[1]Department of Radiation Oncology, Kinki University School of Medicine

INTRODUCTION: We attempted to evaluate the uptake capacity of ^{10}B from a ^{10}B -carrier under aerobic and hypoxic conditions in cultured head and neck tumor cell lines using the two common ^{10}B-carriers, *L-para-boronophenylalanine-^{10}B* (*BPA*, $C_9H_{12}{}^{10}BNO_4$) or *sodium mercaptoundecahydrododecaborate-^{10}B* (*sodium borocaptate-^{10}B, BSH*, $Na_2{}^{10}B_{12}H_{11}SH$) as a ^{10}B-carrier. Further, the effect of the *p53* status of intratumor cells on the uptake capacity using two different tumor cell lines identical in genetic background except for *p53* status was also evaluated.

MATERIALS AND METHODS: Cultured human head and neck squamous cell carcinoma cell line transfected with mutant *TP53* (SAS/*mp53*), or with a *neo* vector as a control (SAS/*neo*) was incubated with *BPA* or *BSH* as a ^{10}B-carrier at the ^{10}B concentration of 60 *ppm* for 24 hours under aerobic (20.7 % of oxygen) or hypoxic (0.28 % of oxygen) conditions. Immediately after incubation, cultured tumor cells received reactor thermal neutron beams, and a cell survival assay was performed. ^{10}B concentration of cultured SAS/*neo* or SAS/*mp53* cells incubated under aerobic or hypoxic conditions was determined with a thermal neutron guide tube.

RESULTS: Hypoxic incubation significantly decreased ^{10}B concentration of cultured cells with a clearer tendency observed following *BPA* than *BSH* treatment in both SAS/*neo* and SAS/*mp53* cells. In both tumor cells, the aerobic incubation with *BPA* produced significantly higher ^{10}B concentrations than with *BSH*. However, after the hypoxic incubation with *BPA*, the ^{10}B concentrations were significantly lower than those with *BSH*. The ^{10}B concentrations after aerobic incubation with *BPA* or *BSH* in SAS/*neo* were higher than in SAS/*mp53* tumor cells although not significantly. However, the ^{10}B concentrations after hypoxic incubation with *BPA* in SAS/*neo* tumor cells were lower than those in SAS/*mp53* tumor cells again without significant differences. Those after hypoxic incubation with *BSH* in both SAS/*neo* and SAS/*mp53* cells were almost the same each other. Following neutron beam irradiation, SAS/*mp53* cells showed significantly higher relative biological effectiveness values than SAS/*neo* cells because of the significantly lower radio-sensitivity of SAS/*mp53* to γ-rays than SAS/*neo* cells.

DISCUSSION: Solid tumors are composed of proliferating (P) and quiescent (Q) tumor cell populations, and human tumors are thought to contain a high proportion of Q cell population [1]. The presence of Q cell population is partly due to hypoxia and the depletion of nutrition in the tumor core, which may be another consequence resulting from poor vascular supply [1]. As Q cell populations have been shown to have a much larger hypoxic fraction (HF) than total (= P + Q) cell populations [1], and Q tumor cell populations also showed a significantly lower sensitivity to the boron neutron capture reaction than the total cell populations [1], it follows that hypoxic tumor cells probably exhibit less uptake capacity of ^{10}B from a ^{10}B -carrier than aerobic cells [1].

It has been thought that the intracellular distribution of ^{10}B from BSH was mostly dependent on the diffusion of the drug, and that ^{10}B from BPA is more dependent on the ability of the cells to take up ^{10}B. On the other hand, Q cell populations were shown to have a much larger hypoxic fraction than total cell populations [1], and have a lower uptake capacity than the total cell population [1]. Thus, it follows that hypoxic cells are thought to exhibit less uptake than aerobic cells, and that the distribution of ^{10}B from ^{10}B-carriers into hypoxic cells is more dependent on the diffusion of the drugs than on the cellular uptake of the cells. SAS/*neo* cells have a functional *p53* protein and SAS/*mp53* cells express a dominant negative *p53* protein. Thus, it was thought to be reasonable that SAS/*neo* showed a significantly clearer decrease in the ^{10}B uptake capacity as a response to hypoxic stress than SAS/*mp53* cells. Further, hypoxic cells are thought to show less ^{10}B uptake capacity than aerobic cell in general. Therefore, it was also reasonable that the ^{10}B uptake capacity from BPA was significantly decreased through hypoxic stress compared with that from BSH because of the more dependency on the ability of cells to take up ^{10}B from a^{10}B-carrier in the use of BPA than BSH, resulting in lower ^{10}B concentrations after hypoxic incubation with BPA than BSH in both SAS/*neo* and SAS/*mp53* cells.

CONCLUSION: Oxygen pressure during incubation with a ^{10}B-carrier had a critical impact on the ^{10}B uptake of cultured tumor cells with a more clearly tendency with the use of *BPA* than *BSH* and in SAS/*neo* than in SAS/*mp53* tumor cells [2].

REFERENCES:

[1] S. Masunaga, *et al.*, J Radiat Res **43** (2002) 11-25.
[2] S. Masunaga, *et al.*, Int J Radiat Biol Phys **92(4)** (2016) 187-94. doi: 10.3109/09553002.2016.1137104. Epub 2016 Feb 18.

PR7-2 Design and Synthesis of Pepducin-BSH Conjugates with Polyamine Linker

A. Isono, T. Hirayama, K. Okuda[1], S. Masunaga[2] and H. Nagasawa[1]

Laboratory of Medicinal & Pharmaceutical Chemistry, Gifu Pharmaceutical University

[1] Laboratory of Organic Chemistry, Kobe Pharmaceutical University

[2] Research Reactor Institute, Kyoto University

INTRODUCTION: Although sodium borocaptate (BSH) has been used clinically as a boron carrier for BNCT, it is impermeable to plasma membrane due to its high hydrophilicity and anionic charges. To improve intracellular uptake of boron-cluster such as BSH to tumor cells, pepducin chemistry was exploited as membrane-permeable carrier. Pepducins are artificial lipopeptides derived from an inner loop domain of G protein-coupled receptors (GPCRs) which enabled anionic molecule such as fluorescein to penetrate membrane directly via flip-flop movement.[1] We focused on the ability of transmembrane translocation of pepducin and new pepducin-BSH hybrid compounds were designed as boron carriers for BNCT (Fig. 1).

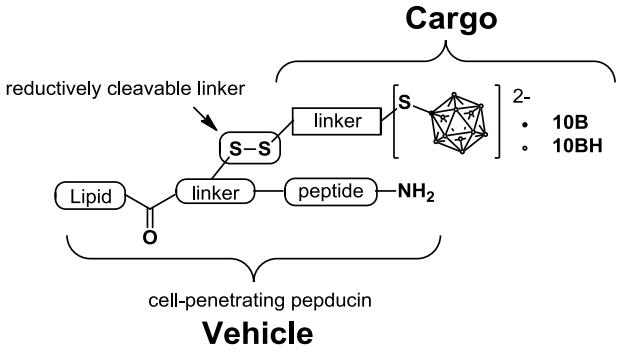


Fig. 1 Design of pepducin-BSH conjugates

Pepducin (Pep) as a vehicle was connected to boron cluster (BS) as a cargo through an appropriate linker. When this molecule is internalized into the cytosol, intracellular glutathione can cleave the disulfide bond (SS) to release the boron cluster cargo into the cytosol. Once BSH is released into cytosol, 10B atoms may be accumulated in the cells due to hydrophilicity of the anionic property. We newly evaluated linker structure in cargo moiety. BSH is membrane impermeable due to its two anionic charges. Polyamine was inserted between BSH moiety and reductively cleavable S-S linker to make the cargo unit neutral or positively charged under physiological condition to increase membrane affinity. Structures of new pepducin-BSH conjugates **1** and **2** with piperazine and linier polyamine linkers respectively were shown in Fig. 2.

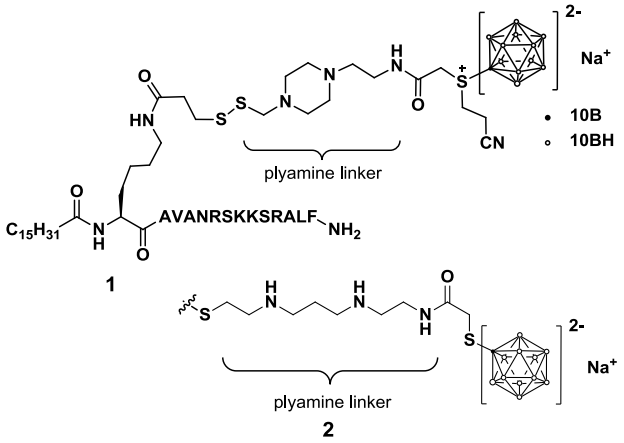


Fig. 2 Structure of new pepducin-BSH conjugates **1** and **2**

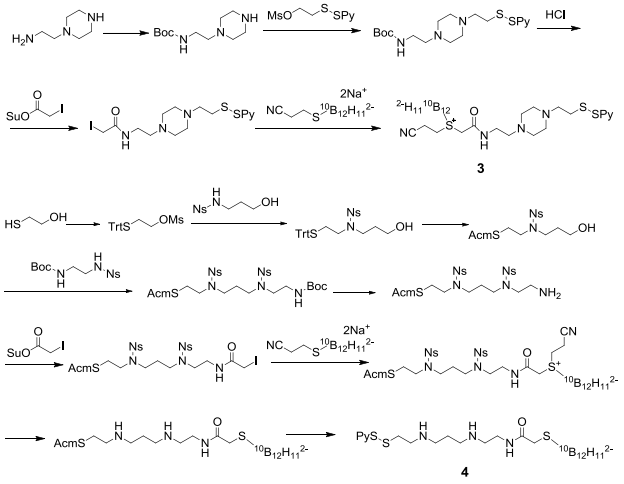


Fig. 3 Synthesys of pluamine linker **3** and **4**

Polyamine linkers **3** and **4** were synthesized in good yields (Fig. 3). Then they were combined with pepducin unit to afford compound **1** and **2** in moderate yields respectively. Now their intracellular uptake into T98G cells is examined by ICP-AES.

REFERENCE:

[1] M. Tsuji *et al.*, *Org. Biomol. Chem.*, **11(**2013), 3030-3037.

PR7-3 UCHL1-HIF-1 Axis-mediated Antioxidant Property of Cancer Cells as a Therapeutic Target for Radiosensitization

H. Harada[1,2,3], R. Nakashima[1,4], Y. Goto[1,4], A. Morinibu[1], M. Kobayashi[1] and S. Masunaga[5].

[1]*Laboratory of Cancer Cell Biology, Department of Genome Dynamics, Radiation Biology Center, Kyoto University.*

[2]*PRESTO, JST.*

[3]*Hakubi Center, Kyoto University.*

[4]*Department of Radiation Oncology and Image-applied Therapy, Graduate School of Medicine, Kyoto University.*

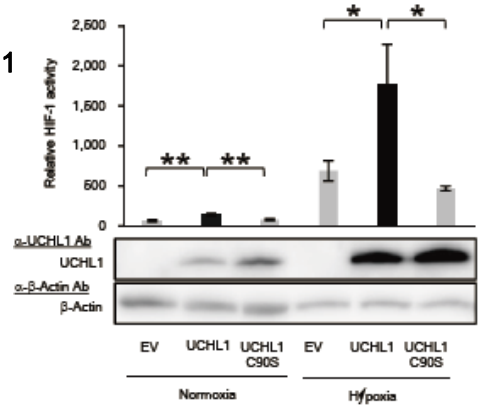
[5]*Research Reactor Institute, Kyoto University.*

INTRODUCTION: Hypoxia-inducible factor 1 (HIF-1) has been recognized as an important mediator of the reprogramming of carbohydrate metabolic pathways from oxidative phosphorylation to accelerated glycolysis. Although this reprogramming has been associated with antioxidant and radioresistant properties of cancer cells, a gene network triggering the HIF-1-mediated reprogramming and a molecular mechanism linking the reprogramming with radioresistance remain to be determined. Here, we show that Ubiquitin C-terminal hydrolase-L1 (UCHL1), which we previously identified as a novel activator of HIF-1, increased the radioresistance of cancer cells by producing an antioxidant, reduced glutathione (GSH), through HIF-1-mediated metabolic reprogramming [1,2].

EXPERIMENTS: EMT6 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM). Media were supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/mL streptomycin. Cells were incubated in a well-humidified incubator with 5% CO2 and 95% air for the normoxic conditions or in a RUSKINN INVIVO2 500 (Ruskinn) for the hypoxic conditions at <0.1% O2. Molecular mechanism behind the UCHL1-mediated upregulation of HIF-1 activity was analyzed *in vitro* by performing Western blotting, luciferase assay. LC/MS-based metabolome analysis to quantify the levels of $^{13}C_6$-labeled lactate, citrate, and isocitrate was performed as described previously[3,4]. Intracellular NADPH levels were quantified using the NADP+/NADPH Quantification Kit (BioVision Inc.) and NADP/NADPH-Glo Assay Kit (Promega) according to the manufacturers' instructions. Intracellular GSH levels were quantified using the ApoGSH Glutathione Colorimetric Detection Kit (BioVision Inc.) and GSH/GSSG-Glo Assay Kit (Promega) according to the manufacturers' instructions. Clonogenic cell survival assays were performed to evaluate effect of UCHL1-overexpression, HIF-1α knockdown, or glucose-6-phosphate dehydrogenase X-linked (G6pdx)-knockdown.

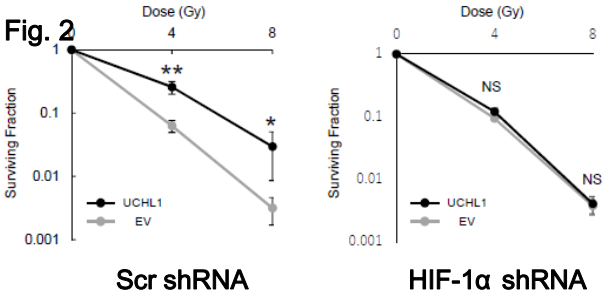
RESULTS: A luciferase assay to monitor HIF-1 activity demonstrated that the overexpression of UCHL1, but not its deubiquitination activity-deficient mutant (UCHL1 C90S), upregulated HIF-1 activity by stabilizing the regulatory subunit of HIF-1 (HIF-1α) in a murine breast cancer cell line, EMT6 (Fig. 1).

Fig. 1



UCHL1 overexpression induced the reprogramming of carbohydrate metabolism from oxidative phosphorylation to glycolysis and increased NADPH levels in a pentose phosphate pathway-dependent manner (data not shown). The UCHL1-mediated reprogramming elevated intracellular levels of GSH, and consequently induced a radioresistant phenotype in a HIF-1-dependent manner (Fig. 2).

Fig. 2



DISCUSSION: These results collectively suggest that cancer cells acquire antioxidant and radioresistant phenotypes through UCHL1-HIF-1-mediated metabolic reprogramming and provide a rational basis for targeting this gene network for radiosensitization.

REFERENCES:

[1] R. Nakashima *et al*. Scientific Reports. in press.
[2] H. Harada. J Radiat Res. 57:99-105. 2016.
[3] L. Zeng *et al.*, Oncogene, 34:4758-4766. (2015)
[4] Y. Goto *et al*. Nature Communications. 6:6153. (2015).

PR7-4 The Response and Survival of Cancer Cells after BNCR

S. Imamichi[1,2], H. Fujimori[2], S. Masunaga[3] and M. Masutani[1,2, 4]

[1]Division of Boron Neutron Capture Therapy, EPOC, National Cancer Center, [2]Division of Genetics, Research Institute, National Cancer Center
[3]Research Reactor Institute, Kyoto University, [4]Department of Frontier Life Sciences, Nagasaki University Graduate School of Biomedical Sciences

INTRODUCTION: Tumors are consisted of heterogeneous populations including hypoxic cells, cancer stem cells and differentiating cells. Boron neutron capture therapy (BNCT) is a tumor selective therapy that is based on the principle of preferential uptake of 10-boron labeled compound such as boronophenylalanine (BPA) into cancer cells and thermal-neutron irradiation. The nuclear reaction between 10-boron and thermal-neutron generates high LET radiation which causes alpha particle and lithium nuclei in the cells. Boron neutron capture reaction (BNCR) efficiently introduces DNA double strand breaks [1], however, tumor cell killing is affected by various factors [2] including the uptake of boron compounds, thermal neutron fluence. It is therefore important to analyze the responses of various cell lines to BNCR in diversed conditions. We previously observed extensive DNA damage responses including that for DNA strand breaks after BNCR by the observation of remaining γ-H2AX and poly(ADP-ribose) in rat lymphosarcoma model of BNCT [3] and started comprehensive analysis of mRNA expression and proteome of human squamous carcinoma SAS cells after BNCR [4]. In this study, we have investigated and evaluated the cell survival after BNCR or neutron beam irradiation in comparison with the survival of gamma-ray irradiation. Clarifying the radiation sensitivity of cells is important to optimize BNCT achieving high precision as a radiation therapy.

EXPERIMENTS: The neutron-beam irradiation with KUR nuclear reactor was not carried out during FY2016. We therefore analyzed the previously irradiated cell samples and cell survival data prepared before. For obtaining cell survival curves, we used the human oral squamous cancer SAS cells and HSG cells, which have been referred as a radiation standard to evaluate relative biological effectiveness. SAS cells and HSG cells were suspended in the polypropylene vials and incubated for 2 hrs with or without 25 ppm of ^{10}B-BPA [5]. In the previous experiments, neutron-beam irradiations were operated at 1 MW in the KUR facility. Cells were diluted and inoculated into 6 well plates. Thermal neutron fluence and gamma-ray dose were measured with the thermaluminescence dosimeter. These physical radiation doses were measured by the kind help of Drs. Yoshinori Sakurai and Hiroki Tanaka of KUR. ^{10}B concentration was measured by prompt-gamma ray analysis (PGA) as described elsewhere. For gamma-ray irradiation, cells were prepared as described above without adding ^{10}B-BPA to the culture medium Gamma-ray irradiations were operated at National Cancer Center Research Institute (Tokyo) with the ^{137}Cs source and dose rate was approximately 100 cGy/min.

RESULTS: SAS cells and HSG cells after BNCR showed a high radiosensitivity. The surviving curve of HSG cells after BNCR showed a slight two-dimensionality (Fig. 1). This may be due to the experimental condition. The relative biological effectiveness (RBE) of BNCR for HSG and SAS cells was around 2-3 as expected from the literatures.

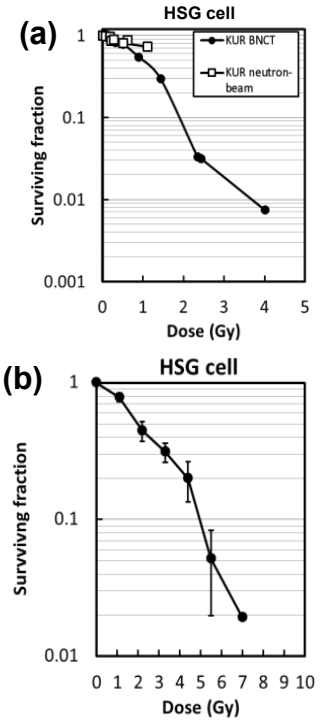


Figure 1: Cell survival after neutron-beam irradiation with/without BPA (a) and after γ-irradiation for HSG cells (b).

REFERENCES:

[1] S.G. Hussein *et al.*, Proc. Montreal Int. Conf. Eds Harvey, Cusson, Geiger, Pearson (U. Mont Press) (1969) 91.
[2] K. Okano *et al.*, Nucl. Instr. and Meth, 186 (1981) 115-120.
[3] M. Masutani et al., Appl. Rad. Iso., 104 (2014) 104-108.
[4] A. Sato *et al.*, Appl. Rad. Iso., 106 (2015) 213-219
[5] R. B. Firestone, in *Table of Isotopes*, 8th ed., edited by V. S. Shirley (Wiley, New York, 1996), Vol.29