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研究課題名（和文）Novel adjuvant method of malignant brain tumor neutron capture therapy with absorbed dose evaluation and tumor localization using composite boron and high-Z element nanoparticles.

研究課題名（英文）Novel adjuvant method of malignant brain tumor neutron capture therapy with absorbed dose evaluation and tumor localization using composite boron and high-Z element nanoparticles.

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研究成果の概要（和文）：我々は、ホウ素ナノ粒子を、微粒子のカスケード超音波分散／破壊によって合成した。加速器中性子源の有効性を、ヒトおよび動物細胞株を用いて証明した。ホウ素に関連した吸収線量計算方法を試験し、独特の公式を開発した。薬物送達システムは標的ホウ素化合物の開発に極めて重要であるため、動物実験においてリポソーム送達を標準的な低分子量ホウ素化合物と比較した。 我々の方法は、加速器ホウ素中性子捕捉療法においてホウ素と高Z元素の組み合わせを用いることによって吸収線量を決定することを可能するので、将来的に我々のホウ素線量推定方法の将来の実施は、シンチグラフィーまたは同位体スキャンを通して実現することが可能となる。

研究成果の学術的意義や社会的意義
In Japan, cancer is one of major reasons for morbidity and mortality, and the number of patients with malignant tumors is increasing year after year. The development of new forms of boron agents will move forward BNCT research and its future clinical application to extend life of cacner patients.

研究成果の概要（英文）：Boron nanoparticles were synthesized by cascade ultrasonic dispersion / destruction of microparticles. The efficacy of the accelerator-based neutron source was proven using human and animal cells lines. Boron-related absorbed dose calculation method was tested and the unique formula was developed. As drug delivery system was crucial for targeted boron compound development, liposomal delivery was compared to standard low-molecular boron compounds in animal experiments. Our method allows in situ absorbed dose calculation using combination of boron and a High-Z element activation in the accelerator-based boron neutron capture therapy. Future implementation of our boron dose estimation method might be realized through modification of scintigraphy or isotope scanning.

研究分野：radiation oncology

キーワード：glioma　nanoparticles　radiotherapy　radiodiagnosis　BNCT

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

Boron-neutron capture therapy (BNCT), a unique form of adjuvant cancer therapy for various malignancies, has been shown to be effective and extends life expectancy in patients with glioblastoma, the most aggressive and rapidly growing glioma [1]. The use of an accelerator instead of a nuclear reactor to produce neutrons makes it possible to place the treatment facility in medical institutions. The neutron capture reaction with alpha-particle release takes place within tumor cells, and, in comparison with other radiotherapy methods, makes it impossible to measure the absorbed dose directly.

Golden foil activation after neutron irradiation is measured for neutron flux estimation [2], as after neutron capture reaction radioactive ^{198}Au isotope with a half-life of 2.7 days is accumulated in the foil. Such a method had been used to evaluate the boron-related absorbed dose in experiments on BNCT. Though, golden foils can provide only approximate data on absorbed dose after gold activation in proximity to the irradiation target. We proposed to use the neutron activation properties of high-Z elements, such as gold, by placing the gold-containing compound inside tumor cells in the form of composite boron and gold nanoparticles for in situ boron-related absorbed dose evaluation and perspective post-radiation malignant brain tumor localization by activated isotope scanning.

２．研究の目的

To develop nanosized boron and gold compounds and perform initial feasibility evaluation for in vitro and in vivo experiments. To evaluate the efficacy of accelerator-based neutron capture therapy using boron compounds and compare with reactor-based BNCT results, previously obtained at a nuclear reactor. To selection most appropriate compounds, evaluate toxicity, efficacy of activation after neutron irradiation, absorbed dose calculation and post-radiation malignant tumor cells localization by activated isotope scanning.

As we could face certain difficulties related with time-consuming and complex process of synthesis of boron and gold nanoparticles, facing unexpected challenges could delay the planned experiments, we planned alternative ways of the study development, including boron-hyaluronic acid compounds development, boron dose calculation methods, gold nanoparticle and liposome selective delivery for BNCT.

３．研究の方法

Boron nanoparticles synthesis: Cascade ultrasonic dispersion / destruction of elemental boron microparticles in an aqueous solution was performed in cooperation with the research group from the Enikolopov Institute of Synthetic Polymeric Materials.

Gold nanoparticles synthesis: gold consolidation on the surface of metallic boron nanoparticles used as crystal basis for the consolidation in several stabilizing solutions was attempted. Additionally, gold nanoparticles were obtained from Winered Chemical, Co. Ltd., (Tokyo, Japan) and Nanoprobes, Inc. (Yaphank, NY, USA).

ICP-AES: Boron and gold concentration in initial samples and accumulation in tumor cells was evaluated by inductively coupled plasma atomic emission spectroscopy at Tsukuba University and Budker Institute of Nuclear Physics using ICP-8100 and ICPE-9800 atomic emission spectrometers (Shimadzu, Japan).

Cell lines: Chinese hamster ovary cells CHO-K1, Chinese hamster lung fibroblasts V79 and human glioma U251MG, T98G and U87MG cells were purchased from the Institute of Cytology of the Russian Academy of Sciences (St.-Petersburg, Russian Federation). CHO-K1, V79, U251MG, and T98G cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (SIGMA 17633 with L-glutamine and 25 mH HEPES, without sodium bicarbonate), supplemented with 10% fetal bovine serum (Thermo scientific HyClone SV30160.03 HyClone UK Ltd.) and gentamicin 50 μg / ml (Dalkhimpharm, Khabarovsk, Russia). U87MG cells were cultivated in DMEM / F12 (1:1) (Biolot, St.-Petersburg, Russia) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and gentamicin 50 μg / ml (Dalkhimpharm, Khabarovsk, Russia). All cells were cultivated at 37˚C in 5% CO_2 atmosphere. Cytotoxicity analysis with MTS assay was performed as described previously [3] using Cell Titer 96 AQueous One Solution (Promega, WI, USA).

Animal experiments were separated according to the stages and analyzed compounds and, therefore, performed at several irradiation facilities in collaboration with Novosibirsk State University, Budker Institute of Nuclear Physics., and Heavy Ion Medical Accelerator in Chiba. Animal experiment protocols were approved by the ethics committees of all participated organizations. Subcutaneous U87MG model was obtained in SCID mice, and F98 intracranial model was obtained in Fisher (F344) rats by methods based on previously published protocols [4].

BPA and BSH: p-boronophenylalanine and borocaptate sodium were purchased from KATCHEM Ltd. (Praha, Czech Republic). The enrichment of ^{10}B was ≥99.6%. 500mg of BPA was mixed with 1100 mg of fructose, 15 ml of H_2O (Milli-Q water) and 2.7ml of 1M NaOH, neutralized with HCl to pH=7.2. Fructose-BPA final concentration was 1100μg of ^{10}B/mL [5].

Transmission electron microscopy (TEM): The size and shape of nanoparticles were studied using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at an accelerating voltage of 120 kV.

Irradiation experiments: a series of irradiation experiments at different facilities have been performed. CHO-K1, V79, U251MG, and T98G cells were irradiated after incubation with 0, 10, 20 and 40 ppm of BPA or boric acid as

boron compounds. T98G cells were additionally incubated with nanoparticles and boron compounds with similar concentrations of boron (0, 10, 20 and 40 ppm). The samples were irradiated in vials with 1 ml of the medium with a boron compound. T98G cells incubated in BPA only without gold were used as controls. To achieve 6mAh of the total irradiation, the samples were placed under the lithium-producing target of the accelerator and irradiated 2.0 MeV proton energy and up to 5 mA proton current. Gamma spectrometer was used to measure gold activation.

Alternatively, SCID mice with subcutaneous models of U87 cells in the right thighs were injected BPA (350 mg/kg), BSH (100 mg/kg), or liposomes with BSH (100 mg/kg). Bodies of the animals were sheltered with lithium-containing plastic, legs with tumors were irradiated with epithermal neutrons with a proton beam current integral of 3 mA/hour and energy of 2.05 MeV at the Budker Institute of Nuclear Physics. Tumor sizes and animal life expectancy were evaluated after irradiation.

Colony-forming assay (CF-assay) was done to check cell proliferation according to the protocol [6]. 50 and over cell-colonies were calculated. The significance was verified by one-way analysis of variance (ANOVA).

４．研究成果

A novel polyborate fragment synthesis along the whole chain of the polysaccharide hyaluronic acid (HA) was conducted. High pressure and deformatory solid-state conditions led to polymolecular system formation with association of phase-specific transition components into a distinct microscopic organization. We found out by Fourier transform infrared spectroscopy that HA and polyborates coalesced into a network of cyclic polychelate complexes. In this system, HA acts as a ligand using carboxylic and hydroxyl proton donor groups to link oxygen atoms in B–O–B bonds and borate-anions B–O(−): O–H···O, O–H···(−)O. With free electron pairs in heteroatoms –O(:)···B, –N(:)···B, HA acts simultaneously to donate electrons. Nuclear magnetic resonance (NMR) with 13C and 1H revealed a preserved complex interaction after both solubilizing and attenuating the HA-polyborate system (Figure.1). This water-stable, cheap and easy to make HA-polyborate complex carries some industrial potential due to its easily scaled synthesis. Thus, these compounds might be more attractive than current approaches in BNCT.

Boron nanoparticles were synthesized by a cascade ultrasonic dispersion / destruction of elemental boron microparticles in an aqueous solution and visualized by transmission electronic microscopy (TEM, Figure 2).

In irradiation experiments with CHO-K1, V79, and U251MG cells incubated with boric acid and bombarded with epithermal neutrons for several hours under a lithium neutron-producing target, boron-loaded cells had their survival curves normalized to control cells to remove the effect of fast neutron and gamma dose components and improve fit to the LQ model. Three cell lines doped with boron showed excellent colony forming ability, demonstrating the efficacy of BNCT (Figure 3). The difference between treated cells and controls was significant in all cases ($P < 0.01$). In vitro experiments on neutron capture therapy were developed into animal models and further development of accelerator-based BNCT was promoted.

BPA uptake by the cells was evaluated after 24-hour incubation with 20, 40 and 80 ppm of boron (Figure 3). In experiments with T98G cells irradiation with gold nanoparticles and boron dose calculation, colony forming assay showed the efficacy of BNCT (Figure 4).

Radiobiological parameters, such as α' and β' values and C10 (instead of D10) was calculated by solving the following quadratic equation:

$$\alpha' C + \beta' C^2 + ln(SF) = 0,$$

where C represented ^{10}B concentration in cells, and in cases with linear survival curves (where β' = 0) equaled ln(SF)/ α', otherwise:

$$C = \frac{-\alpha' \pm \sqrt{\alpha'^2 - 4\beta' ln(SF)}}{2\beta'};$$

positive values of C were used.
The following formula have been developed for boron dose calculation using activation of gold nanoparticles.

$$D(GyE) = \frac{k*N*n}{m}, \text{ where}$$

D – boron dose, GyE;
N – number of activated gold atoms;
n – boron concentration, ppm;
m – mass of gold, g;
k – coefficient, which depends on the depth (cm) to the sample in the phantom.

As the stability of the compounds during high-Z element consolidation differed in the experiments, in vivo application was shifted to development of the drug delivery system. Animal tumor model using U87 malignant glioma in SCID mice was successfully created. Liposomal delivery of the compounds was implemented and compared with the low molecular boron compound delivery at that stage.

Thus, in animal experiments, U87 cells were subcutaneously injected into SCID mice. In the post-BPA group on the 32nd day after irradiation, significant ($p \leq 0.05$) tumor growth slowdown of 59.7% compared to irradiated controls was seen and a 5.6 times reduction in tumor growth was seen compared to the untreated controls. Over the day 32 to day 46 timepoints, tumor growth was 18% slower compared to the irradiated controls and 2.6 times slower than in non-irradiated controls. In the post-BSH injection group after further neutron irradiation, a significant ($p \leq 0.05$) slowing of tumor growth compared to the untreated control was observed on the 32nd day after tumor inoculation. No obvious significant differences in tumor volume compared to irradiated controls was observed. On day 46, the volume increase was 52.5% less prominent compared to the untreated controls. Similar results were seen in the liposomal delivery group, i.e., a tumor growth slowing compared to untreated animals without a significant difference in volume increase compared to the irradiated controls. On day 46, the volume increase was 62% less than the untreated controls. BNCT at the accelerator-based neutron source tended to reduce or suspend the growth of human glioblastoma in immunodeficient animals. BPA and liposomal BSH were better in the long term than non-liposomal BSH but no significant therapeutic advantages were seen with liposomal BSH compared to BPA. Further modifications in liposomal boron delivery are being studied to improve this outcome.

To conclude, our experiments showed promising results for the future of accelerator-based BNCT, combination of boron with gold allows for evaluating boron-related absorbed dose, gold doesn't influence the cell survival after neutron irradiation, our method opens a new perspective for boron distribution evaluation and treatment efficacy evaluation using isotope scanning and positron emission tomography.

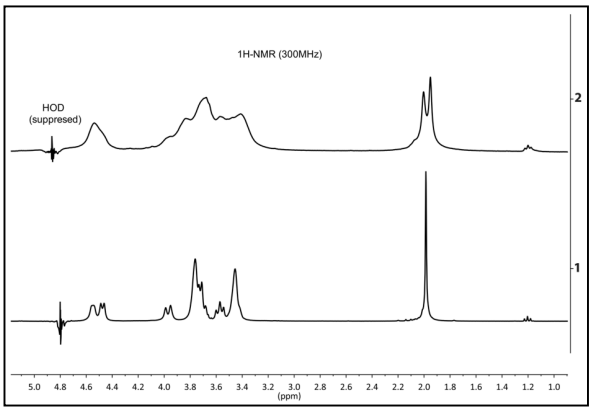

Figure 1. 1H NMR spectra of the alkaline hyaluronic acid (1) and the hyaluronic acid, borax and potassium hydroxide solution in a molar ratio of 1:4:4, respectively (2). pH = 14 in all solutions. The borate formation is indicated by the general broadening of the spectrum and the splitting of the signal of the protons of the acetyl group (about 2 ppm). The triplet with the chemical shift of 1.17 ppm belongs to the methyl group of the ethanol internal standard. (Zelenetskii AN, Uspenskii S, Zaboronok A, et al. Polymers.10(2):181, 2018.)

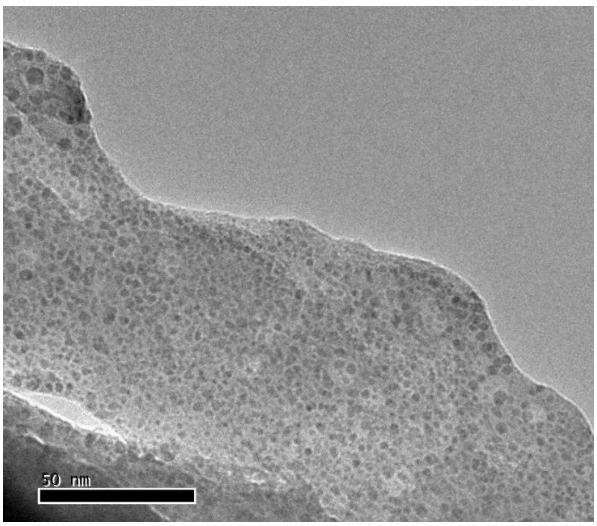

Figure 2. Boron nanoparticles synthesized by a cascade ultrasonic dispersion / destruction of elemental boron microparticles in an aqueous solution.

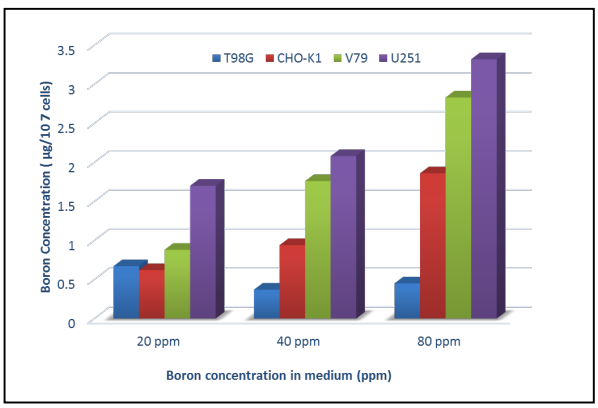

Figure 3. U251 glioma cells showed maximum accumulation of BPA, 24h incubation, ICP-AES (ICPE-9820, Shimadzu, Japan).

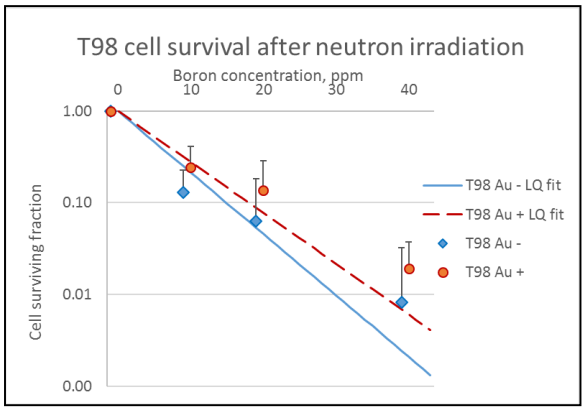

Figure 4. T98G cell survival after incubation with boron and gold and neutron irradiation. Colony forming assay showed the efficacy of BNCT and the curves were fit to the LQ model.

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５．主な発表論文等

〔雑誌論文〕（計　2 件）

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2. Zaboronok A, Taskaev S, Kanygin V, et al. Hybrid gold and boron nanoparticles for treatment and boron dose estimation in boron neutron capture therapy for malignant glioma. The 18th International Congress on Neutron Capture Therapy (ICNCT-18), Taipei, Taiwan. 2018.10.28-11.2
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〔その他〕

1. 15th Kenichi Uemura Award given to Alexander Zaboronok for the second best presentation at the 21st JASMEE Academic Meeting (July 28-29 2018, Tokyo, Japan), the award will be given at the 22nd JASMEE Academic Meeting (August 3-4, 2019, Tokyo, Japan).
2. "Certificate of Appreciation" [**感謝状**] presented to Alexander Zaboronok in appreciation of his outstanding contribution to Workshop "COMBINED APPROACH IN SPONDYLOLISTESIS SURGICAL TREATMENT", Almetyevsk (Kazan), Russian Federation. 2018.09.10-11.
3. "Certificate of Appreciation" [**感謝状**] awarded to Alexander Zaboronok in recognition of exceptional contribution to the 6th Japan Russian Neurosurgical Symposium held in Fukui, Japan, 2018-5.20-22.
4. Zaboronok A. Current state and vision of Boron neutron capture therapy (BNCT). Japan-Russia Joint Symposium on Education and Scientific Development in Medicine 2018, Niigata, Japan. 2018.11.9-10. **招待講演日本脳神経外科国際学会フォーラム** Sammy's Award, 1st Place, given to Alexander Zaboronok for best neurosurgical presentation in English (2017.07.14)

ホームページ等

Alexander ZABORONOK, MD, PhD, Assistant Professor. Boron Neutron Capture Therapy: Current State and Future Perspectives. International Stereotactic Radiosurgery Society Webinar (**招待講演**). April 10, 2019.
Homepage: http://isrsy.org/public/en/courses/webinars/past/
Lecture:http://isrsy.org/public/en/courses/webinars/replay/boron-neutron-capture-therapy-current-state-and-future-perspectives/web_id/57

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