



## BORON-NEUTRON CAPTURE THERAPY IN RUSSIA: PRECLINICAL EVALUATION OF EFFICACY AND PERSPECTIVES OF ITS APPLICATION IN NEURO-ONCOLOGY

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### ABSTRACT

*Boron-neutron capture therapy is a unique form of adjuvant cancer therapy for various malignancies including primary malignant brain tumors, and especially glioblastoma, characterized by the fastest and most aggressive type of growth. The main advantage of boron-neutron capture therapy is the selective destruction of tumor cells without harming normal tissues. Clinical trials have shown that boron-neutron capture therapy is a promising treatment method of various malignancies.*

*The use of nuclear reactors in therapy is unfeasible from a safety standpoint. Therefore, relatively safe accelerator-based epithermal neutron sources for boron-neutron capture therapy are in worldwide development. The accelerator-based epithermal neutron source constructed in Budker Institute of Nuclear Physics in Novosibirsk (Russia) offers a unique opportunity to test this new therapy method.*

*Joint teams of physicists, biologists and medical doctors carry out multiple experiments to assess the efficacy of the accelerator-based epithermal neutron source to be further used in preclinical and clinical trials, the results of which are presented in this article.*

*To determine optimal irradiation conditions and evaluate the effect of boron-neutron capture therapy on tumor cell survival, experiments were conducted on human glioma cell line (U251MG). The cells were incubated in medium with boronophenylalanine and irradiated by epithermal neutron flux. Boron-negative cells, irradiated by neutrons, as well as those cells, which weren't irradiated by neutrons were used as controls. The colony forming assay showed that generated neutron flux doesn't affect cell viability without boron and is effective in treating tumor cells, in which boron is accumulated.*

*Experimental studies on the influence of neutron flux and boron neutron capture therapy on animals depending on the radiation dose were conducted. The study included severe combined immunodeficiency mice pretreated with sodium borocaptate. Control group included animals without boron administration and without irradiation. Dynamic monitoring on the condition of the animals was performed. Evaluation of tissue damage was carried out using histological examinations. The result of the experiment revealed that the neutron flux in therapeutic doses doesn't affect the condition of the animals, and the dose to healthy tissues of immunodeficient mice during irradiation is tolerant.*

*The study present a novel method of boron drug delivery to the tumor tissue by means of pegylated liposomes with a fluorescent label, which can increase the efficacy of boron-neutron capture therapy and determine the localization of the compound in tissues.*

**KEYWORDS:** boron-neutron capture therapy, accelerator-based epithermal neutron source, glioma, colony forming assay, boron delivery agents.

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### INTRODUCTION

The most advanced technologies in neurosurgery, particularly radio- and chemotherapy, are not able to significantly extend life of patients with

glioblastoma, the most malignant primary brain tumor [Bush N et al., 2016]. The limiting factor for therapy is metastatic invasion deep into normal brain tissue, even in cases of perfect treatment, and this limits the median survival of patients with glioblastoma to approximately 14.6 months after the diagnosis [Stupp R et al., 2009] with a 5-year survival ratio of 5% [Delgado-López P, Corrales-García E, 2016]. Effective tumor control must be targeted to the single cell level, as glioblastomal cells may migrate large distances from the primary tumor mass and form secondary tumors with poor prognosis [Dudu V et al., 2012; Baker G et al., 2014; Zaboronok A et al., 2014].

Boron neutron capture therapy is a unique type of single cell-targeting radiation therapy used in the treatment of invasive tumors. It was firstly proposed by G.L. Locher [Locher G, 1936], but only recently has become practical as a therapy method in medical practice. The method is based on the interaction of two relatively harmless components: a boron-10 nucleus and a thermal neutron. This two-step process is comprised of a compound containing an injectable, stable isotope of boron-10 that accumulates in tumor cells followed by irradiation of the tumor region with epithermal neutrons. Boron-10 nucleus captures neutron in the tumor cell, resulting in a reaction, the energy of which leads to the cell destruction. The use of boron-10 carriers, which are selectively accumulated in tumor cells, allows for specific destruction of tumor cells while sparing normal, unchanged cells. As a result of neutron absorption by boron-10, unstable boron-11 nuclei are formed, which instantaneously decay into alpha-particles (helium nucleus) and lithium nuclei (Fig. 1).

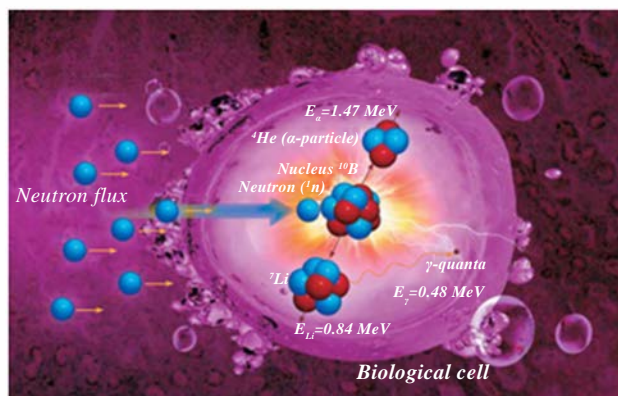


FIGURE 1. Schematic diagram of neutron capture by boron-10 in the tumor cell and the subsequent release of energy

Contained within the target cell, alpha particles and lithium nuclei transfer their energy of 2.31 MeV to tumor cell structures, including DNA. Thus, single-cell targeting protects surrounding normal brain tissue since the path of the particle is less than the size of a single cell (around 7 to 5 microns). In boron neutron capture therapy, a micronized nuclear reaction is initiated with the formation of alpha radiation at a cellular level, which is unique among other types of radiation therapy.

Boron neutron capture therapy has been tested worldwide in both clinical and preclinical settings. The University of Tsukuba carried out a pilot clinical trial of boron neutron capture therapy, showing up to a 2-fold increase in median overall survival in patients with confirmed glioblastoma, including one patient who survived for over 9 years after initial boron neutron capture therapy [Yamamoto T et al., 2009b; Matsuda M et al 2009; 2011; Nakai K et al., 2011]. Nuclear research reactor JRR-4 (Tokai, Japan) was used as a neutron source. In spite of the promising results, however, the use of the nuclear reactor was frequently interrupted due to regular inspections for safety, and it was finally decommissioned after the Fukushima nuclear incident in 2011.

Inherent safety problems with reactors forced creativity in development of alternative neutron sources, which turned out to be proton accelerators with lithium or beryllium targets that produced neutrons without dangerous nuclear reactions. In the Russia, such an accelerator was constructed at Budker Institute of Nuclear Physics in Novosibirsk [Taskaev S, 2015]. The source of epithermal neutrons was developed on the base of a new type of proton accelerator – a vacuum-insulated tandem accelerator [Bayanov B et al., 1998; Ivanov A et al., 2016] and lithium target [Bayanov B et al., 2006]. At this point in time, this facility is unique in the world at providing neutrons of a desirable energy range for experiments. The accelerator is specially designed to be mounted in oncological centers and passed all preliminary tests [Iarullina A et al., 2015; Kanigin V et al., 2015].

Present study, conducted at Budker Institute of Nuclear Physics, was aimed to evaluate the efficacy of the accelerator-based neutron source in cell and animal experiments, as well as the possibilities to utilize that technology in the treatment of patients with gliomas and other malignant tumors.



## MATERIAL AND METHODS

**Human glioma cell line:** U251MG cells, purchased from the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia), were cultured in Iscove's modified Dulbecco's medium (L-glutamine and 25mM HEPES buffer, without sodium bicarbonate, SIGMA 17633 (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum ThermoScientific HyClone SV30160.03 (HyClone, UK), maintained in 5% CO<sub>2</sub> at 37°C.

**Fructose-boronophenylalanine solution:** Boronophenylalanine was purchased from Katchem Ltd (Czech Republic). The enrichment of <sup>10</sup>B was ≥99.6%. Boronophenylalanine of 500mg was mixed with 1100 mg of fructose, 15 ml of H<sub>2</sub>O (Milli-Q Water, USA) and 2.7ml of 1 M NaOH, neutralized with hydrochloride to pH=7.2. Final concentration of fructose-boronophenylalanine was approximately 1100 μg of <sup>10</sup>B/ml [Yoshino K et al., 1989]. Fructose-boronophenylalanine complex was added according to the previously evaluated boron accumulation in cell lines [Yoshida F et al., 2002].

**Cytotoxicity assay:** Cytotoxicity evaluation was performed to determine reasonable and maximum non-toxic boron concentrations by the method described in our previous [Zaboronok A et al., 2013]. Minimal essential medium of 100μl with 4×10<sup>4</sup> of cells was placed in each well of 96-well plates and incubated for 24 h. The medium was replaced by minimal essential medium with boronophenylalanine (0-320μg boron/ml) and further incubated for 24 hours. Minimal essential medium with boronophenylalanine was removed; the cells were washed with physiological solution, 2 ml of [3-(4.5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl) -2H] tetrazolium solution with phenazine methosulfate (Cell Titer 96® Aqueous One Solution, Promega, USA) was mixed with 10 ml of minimal essential medium, and added in the amount of 100 μl to each well. The plates were incubated for 2 hours, and absorption at 490 nm was assessed using a Bio-Rad Model 2550 EIA plate reader (Bio-Rad Inc., Hercules, CA, USA).

**Irradiation experiments:** Neutron irradiation was carried out at Budker Institute of Nuclear Physics (Novosibirsk, Russia). After 24-hour incubation with boronophenylalanine containing boron-10 (40 μg/ml), human glioma cells in the medium with bo-

ronophenylalanine were placed in plastic vials in a phantom made of organic glass under the lithium target of the tandem accelerator with vacuum insulation (Fig. 2). The irradiation was performed for 1-3 hours with the following accelerator settings: 2.0 MeV proton energy, 1-3 mA proton current. Epithermal neutron flux was set up to 3×10<sup>8</sup>cm<sup>-2</sup>s<sup>-1</sup>.

**Colony forming assay:** After irradiation, the cells were counted, diluted and seeded into round 6 cm dishes for colony formation assessment according to the protocol used in our previous studies [Franken N et al., 2006; Zaboronok A et al., 2013]. In short, 2 weeks after the irradiation the dishes were washed with phosphate-buffered saline, the cells were fixed with glutaraldehyde, stained with crystal violet, and dried. The colonies of greater than 50 cells were counted for each sample and each irradiation dose. The results were compared to the controls irradiated without boron. Data are presented as mean ± standard deviations; p values were determined by one-way variance analysis (ANOVA).

**Experiments on animals:** All animal experi-



**FIGURE 2.** Process of specimen irradiation. U251MG cells were placed in plastic vials (A) in a phantom made of organic glass under the lithium target (B) of the tandem accelerator with vacuum insulation (C)

ments were approved by Inter-institutional committee on bioethics of the Institute of Cytology and Genetics SB RAS and correspond to the principles of Guide for the Care and Use of Laboratory Animals [NIH USA, No 85-23, rev. 1985]. The study was conducted on 14 immunodeficient severe combined immunodeficiencies of mice were used (SHO-Prkdc<sup>scid</sup>Hrhr, USA). Six of them were intraperitoneally injected with sodium borocaptate at a dose of 200 mg/kg (mouse body weight was approximately 30 g) and subjected to irradiation under anesthesia with 0.1% Domitor and 99.9% Zoletil. Six mice were irradiated without boron and 2 mice were kept for control without sodium borocaptate administration or irradiation. Mice were placed into a container made of 25 mm lithium-polyethylene plates. In the center of the container an aperture for exposure 10 cm in diameter was made and filled with 2 cm of paraffin. In such a container, the absorption dose under the aperture was 2 times higher, than under the polyethylene, which helps to decrease the irradiation dose to radiation sensitive areas of the animal body, that are normally shielded (Fig. 3).

*Experiment with irradiation of animals:* The irradiation was performed in 2 hours after the sodium borocaptate injection. In every irradiation group with or without sodium borocaptate injection, 2 mice were irradiated within 30 minutes, 2 – 60 minutes, and 2 – 90 minutes. Such time intervals are typical for boron-neutron capture therapy. Between the irradiation sessions, body temperature control was performed using a digital thermometer. Mean mouse body temperature was around 27-29°C. Irradiation doses received by

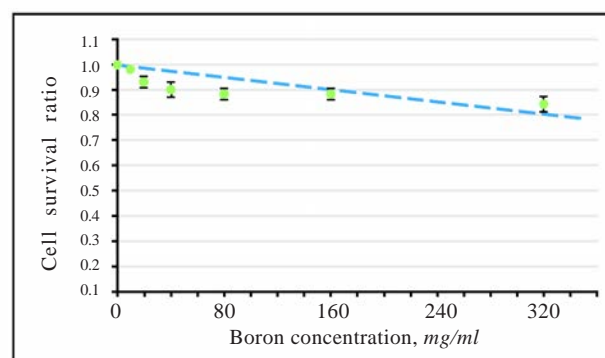


**FIGURE 3.** Animal positioning. Mice were radially placed in the container with their heads inside

mice organs did not exceed 5.7 Gy-Eq in mice with injected sodium borocaptate and 2 Gy-Eq in mice without sodium borocaptate injection. Such doses were accounted as tolerable, i.e. the exposure could not cause any lethal radiation damage. After irradiation, mice were kept in SPF-vivarium of the Institute of Cytology and Genetics SB RAS by same-sex family groups of 2-5 species in the individually ventilated cells of the system OptiMice (Animal Care Systems, USA) under controlled conditions (at 22-26°C, relative humidity of 30-60% and light regime light/dark 14/10 h with the sunrise at 01:00). The observation period lasted 1 month. During the experiment the condition of mice was daily registered. Particularly, changes of the condition of the skin, motor activity and behavior were assessed. To assess the effects of different doses of radiation on animal bodies after the observation period, euthanasia was performed by approved craniocervical dislocation. At the end of the experiment euthanasia was performed with CO<sub>2</sub> overdosage accompanied by cervical dislocation. Animal organs (kidney, liver, brain, heart, spleen) were dissected and fixed in formaldehyde solution.

## RESULTS AND DISCUSSION

*Boronophenylalanine cytotoxicity:* It was established that boronophenylalanine was found to be well tolerated by U251MG cells and cytotoxicity was found to be dose-dependent (Fig. 4). While analyzing various boron concentrations, the lowest toxicity was observed with 10 to 40 µg/ml of boron, showing a maximum proliferation rate of over 90%. The lowest proliferation rate of 0.844±0.028, which



**FIGURE 4.** Boronophenylalanine cytotoxicity towards U251 glioma cells. Cell survival depending on boron concentration (0-320 µg/ml)



can also be considered as relatively high, was observed at the maximum concentration of 320  $\mu\text{g/ml}$ . The cytotoxicity assay proved the non-toxic nature of boronophenylalanine. Thus, for the neutron source efficacy evaluation 40  $\mu\text{g/ml}$  of boronophenylalanine was used to minimize the effect of the drug on glioma cell colony formation.

**Glioma cell survival after neutron irradiation with boronophenylalanine:** Colony forming abilities of U251MG cells with boronophenylalanine (40  $\mu\text{g/ml}$ ) and without the compound are presented in figure 5 and 6. Neutron capture by boronophenylalanine decreased tumor cell survival exponentially depending on the amount of delivered neutron radiation, proving its therapeutic effect. The most significant effect was observed at the maximum amount of delivered neutrons.

Survival of U251MG tumor cells, incubated in boronophenylalanine medium, reduced with the increase of neutron flux. Thereat, the increase of

neutron flux showed an insignificant influence on cells without boron. The obtained data comply with previously reported data on the effect of nuclear reactor-based neutron sources.

Colony forming abilities of U251MG cells decreased exponentially and significantly differed from those of the cells irradiated without boronophenylalanine. Irradiation without boron proved to be benign, demonstrating the safety of the accelerator-based neutron source in preclinical *in vitro* experiments.

**Neutron irradiation influence on laboratory animals:** All laboratory animals were alive in 1 month after irradiation. External pathological signs also were not detected.

Morphological study in liver of irradiated animals showed variable moderate or significant focal and subtotal hydropic dystrophy, but with no correlation to the time of exposure and injection of boron-containing compound (Fig.7).

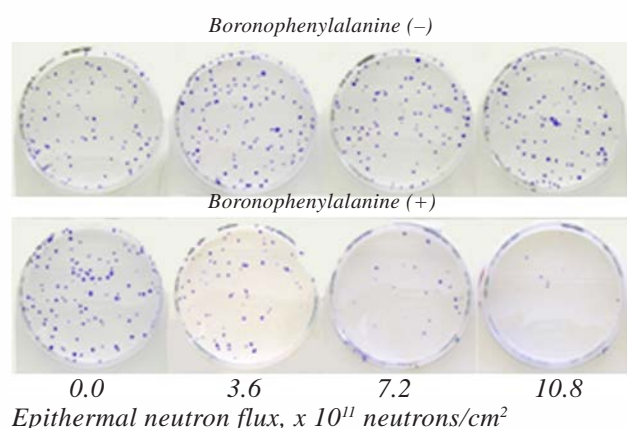
Unlike the irradiated animals, cytoangioarchitectonics of the liver was preserved in control animals (Fig.8).

Morphological examination of the animal kidneys exposed to radiation revealed moderate focal and subtotal hydropic dystrophy in the epithelium of the convoluted tubules without evidence of a correlation with the time of exposure and injection of the boron-containing compound (Fig.9, 10).

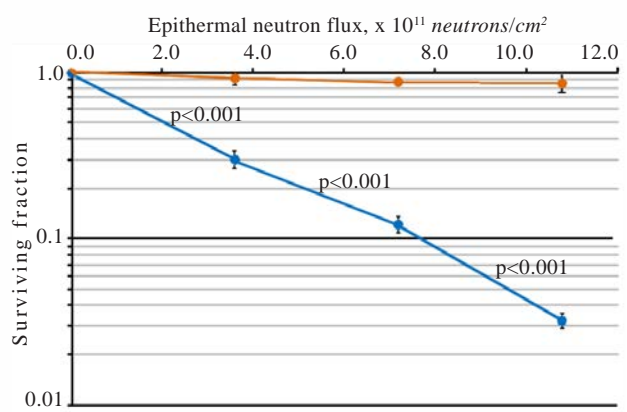
In contrast to irradiated animals, no morphological changes of dystrophic type were found in kidney tissues of control animals (Fig. 11).

Structural changes of hypoplastic character were revealed in red and white pulp of the spleen of experimental animals (Fig. 12).

Cytoangioarchitectonics of various structures of brain and heart of experimental animals under the conditions of neutron irradiation practically didn't differ from that of in intact animals. Thus, the influence of neutron irradiation on the liver and kidneys, appearing in changeable hydropic dystrophy, as well as in hydroplastic shifts of lymphoid tissue in red and white pulp, was found during the morphological analysis. Structural changes were not found in the brain tissues exposed to neutron radiation. Thus, according to the results of own studies, it can be concluded that the dose received by the healthy tissues of immunodeficient mice during irradiation can be accounted as tolerable and can be used in the following animal experi-

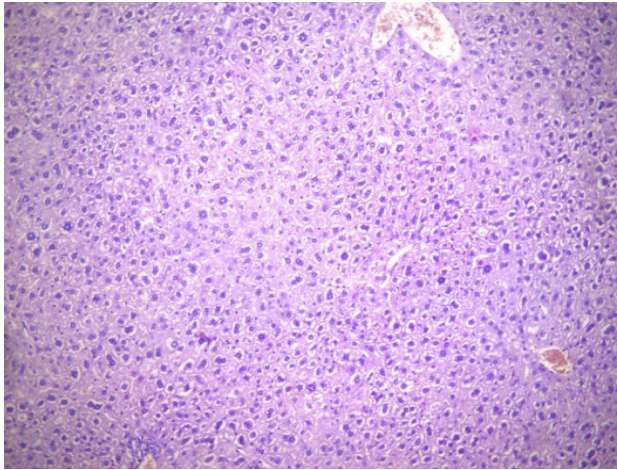


**FIGURE 5.** Colony forming abilities of U251MG cells with and without boronophenylalanine depending on epithermal neutron flux ( $3.6-10.8 \times 10^{11}$  neutrons/cm<sup>2</sup>)

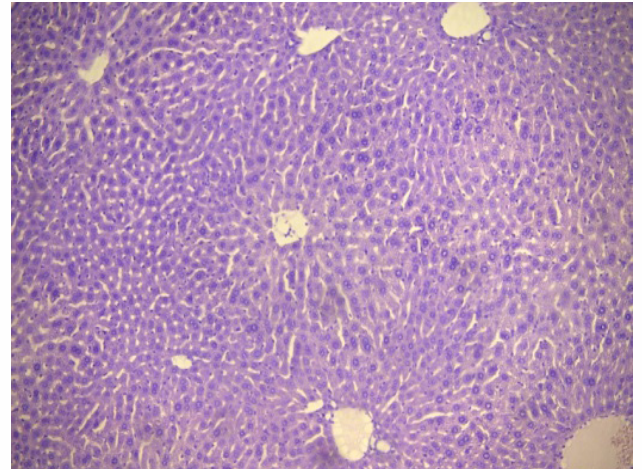


**FIGURE 6.** U251MG cell survival ratio with and without boronophenylalanine depending on neutron flux

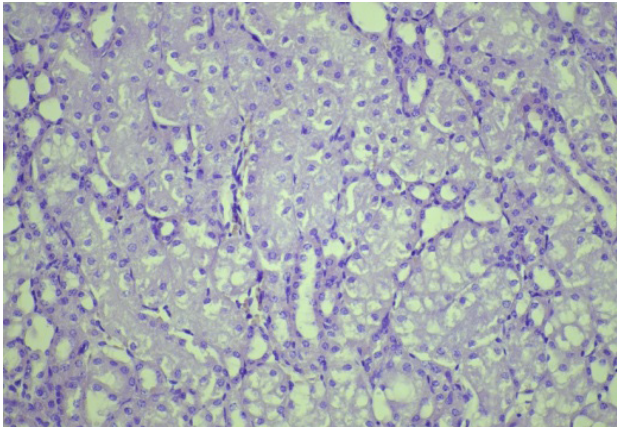




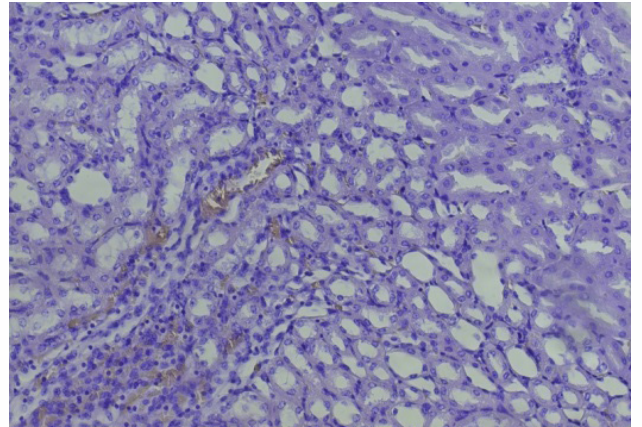
**FIGURE 7.** Most hepatocytes are in a state of hydropic (protein) dystrophy with a large vacuolization and with the presence of fine and eosinophilic granularity in the cytoplasm (X 200)



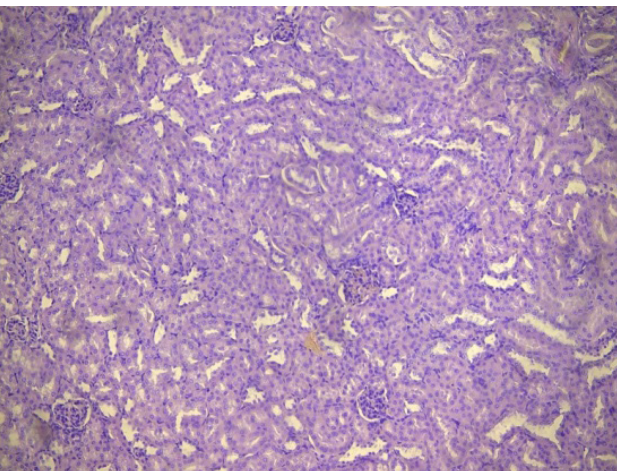
**FIGURE 8.** Hepatocytes with smooth, clearer contours, regular shape and homogeneous cytoplasm. There are individual hepatocytes with mild eosinophilic granularity in cytoplasm (X 100)



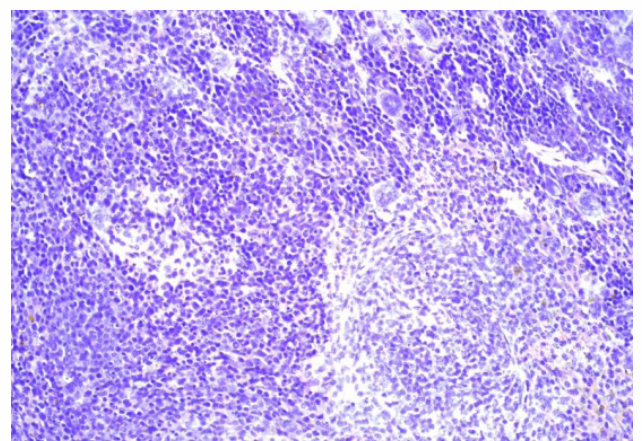
**FIGURE 9.** The epithelium of convoluted tubules with signs of hydropic dystrophy structurally appearing as uneven grainy cytoplasm, its vacuolization and different blurred apical edges (X 100)



**FIGURE 10.** The epithelium of distal tubules is without significant pathological changes. In stroma in the area of pyramids there are foci of minimum lymphoid infiltration (X 100)



**FIGURE 11.** The division into cortex and medulla in kidney tissue is preserved. The glomeruli are lobed; typical morphological structure is preserved. The epithelium of convoluted and distal tubules is without significant pathological changes (X 100)



**FIGURE 12.** The division into red and white pulp is indistinct. The number of follicles is significantly decreased; remaining follicles are in state of atresia. In the latter the division into mantle and marginal zones is absent. Hyperemic sinuses, hemosiderin, small lymphoid cells, and a large number of scattered megakaryocytes are detected in the red pulp (X 100)



ments with orthotopic tumor xenografts.

**Boron-containing compounds:** Clinically-approved boron-containing agents – boronophenylalanine and sodium borocaptate, which were successfully applied in boron-neutron capture therapy for malignant tumors, including head and neck cancer [Barth R et al., 2012, Aihara T et al., 2014; Wang L et al., 2016], melanoma [Menéndez P et al., 2009], brain tumors (glioma) [Yamamoto T et al., 2004, 2008, 2009a; Matsuda M et al., 2009; Matsumura A et al., 2009; Nakai K et al., 2011; Barth R et al., 2012] were used in present study. Boronophenylalanine is noted for its ability to penetrate the blood-brain barrier and specifically accumulate in active tumor cells with tumor/blood ratio of 2.0-4.0 after intravenous infusion (250-900 mg/kg). In contrast to boronophenylalanine, sodium borocaptate does not penetrate an intact blood-brain barrier and is distributed via passive diffusion through tumor-related capillaries, providing a tumor/blood ratio of 0.5-2.0 after intravenous infusion (100mg/kg). Both drugs are available commercially with international shipping restrictions based mostly around the boron isotope. One of the weak points of these compounds remains their selectivity and a small number of boron atoms, and therefore, effective components per molecule. Selectivity of boronophenylalanine is based entirely on increased accumulation of phenylalanine amino-acid in gliomas. On the other hand, sodium borocaptate, being unable to be selectively accumulated in migrated single tumor cells, completely relies on passive tumor tissue penetration.

In previous experiments we tested a water-soluble polymer-based boron-hyaluronic acid compound, produced by a solid-state synthesis from the mixture of hyaluronic acid powder and borax [Zaboronok A et al., 2015]. The targeting of tumor cells was suggested to be effected through CD44 receptors, which are widely presented in tumors and especially in gliomas. The compound showed high stability in water solution and had a high ratio of boron atoms to total molecules but exhibited poor accumulation in a C6 rat glioma model, showing the necessity for its further modification to be used in animal experiments.

Though boronophenylalanine and sodium borocaptate showed their efficacy in preclinical [Yoshida T et al., 2004; 2014] and clinical trials [Chanana A et al., 1999; Wittig A et al., 2002; Busse P et al., 2003; Capala J et al., 2003; Diaz A et al., 2003; Joensuu H et al., 2003; Burian J et al., 2004;

Coderre J et al., 2004; Yamamoto T et al., 2004; Miyatake S et al., 2005; Yamamoto T et al., 2008; 2009a,b], and new clinical trials protocols had been developed, however, more selective compounds would be beneficial to provide better tumor cell killing and prolong overall survival in patients. Present study was focused on the development of a substance, which could be accumulated by tumor cells in sufficient therapeutic concentration (the optimal tumor/normal tissue boron-10 ratio was set to 3:1) [Barth R et al., 2012]. We were also faced with more serious challenge of both penetrating the blood-brain barrier and selecting only highly infiltrative, molecularly heterogeneous glioma cells [Byvaltsev V et al., 2015].

To improve the efficiency of therapy, we have proposed to deliver boron-containing agents into brain tumor cells by pegylated liposomes with a fluorescent label that allows for estimation of localization of the compounds in the target tissues [Tasakaev S et al., 2016]. A potential benefit from the use of liposomes is their ability to penetrate deeply into a cell. Liposomes are able to contain both hydrophilic and amphiphilic hydrophobic substances, which allows their usage as carriers of various boron-containing compounds. Furthermore, such a carrier type can be effectively used in the treatment of brain tumors, as liposomes allow the transport of boron compounds through the blood-brain barrier. Polyethylene glycol allows for passive targeting of tumor tissue while limiting uptake by cells of the reticuloendothelial system [Nakamura H, 2009].

Therefore, encapsulation of boron-containing compounds into liposomes with polyethylene glycol can improve treatment efficacy and reduce the standard doses of boron drugs to limit treatment toxicity and cost.

There are a plenty of methods for detecting the presence and amount of boron in the tumor mass, but the intracellular penetration of boron can only be detected using various labels. To confirm tumor penetration by boron-containing drugs, fluorescent labels, included in liposomes, can be effectively used. Conducted studies demonstrated the efficacy of fluorescent and confocal microscopy in determining intracellular localization of substances contained in liposomes with a fluorescent label. The simultaneous presence of two different dyes in different parts of the liposomes allows separate localization of substances, included in the lipid membrane, as well as those in the aqueous phase of

the liposomes. This provides a control and a marker for successful drug delivery at the cellular level, minimizing waste. Determining the quantitative ratio of the luminosity inside the cell and the extracellular space is simple and accurate.

The proposed boron-containing drug delivery method will be tested in preclinical studies on boron-neutron capture therapy to determine the optimum concentrations of boron compounds and to select the time interval of the maximum drug concentration in the tumor tissue to optimize boron-neutron capture therapy.

*Boron-neutron capture therapy application in Russia:* The delivery of boron-10 compounds might be beneficial only in case of the presence of accelerator-based neutron source. Along with the development of new boron-containing agents, the

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construction of a patient treatment-oriented accelerator [Aleynik V et al., 2014; Sorokin I, Taskaev S, 2015] and placing it in the specialized center for boron-neutron capture therapy is necessary for developing new treatments of oncological diseases.

Successful experiments on the accelerator-based neutron source conducted at the Budker Institute of Nuclear Physics SB RAS proved the efficacy of neutron source for boron-neutron capture therapy created in Russia. Positive results such as reported here have defined both the main direction of activities by Russian research groups to focus their scientific efforts and the Russian Government authorities to financially support this endeavor in the development of new effective approaches of malignant neoplasm therapy.

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