Boron-neutron capture therapy in Russia: preclinical evaluation of efficacy and perspectives of its application in neurooncology

Zaboronok A.A.[a], Byvaltsev V.A.[b], KanyginV.V.[c, d], Iarullina A.I.[b], Kichigin A.I.[c], Volkova O.Yu.[e], Mechetina L.V.[e], Taskaev S.Yu.[c, d], Muhamadiyarov R.A.[f], Nakai K.[a], Sato E.[a], Yamamoto T.[a], Mathis B.L.[a], Matsumura A.[a]

[a] Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

[b] Irkutsk State Medical University, Irkutsk, Russia

[c] Budker Institute of Nuclear Physics, Novosibirsk, Russia

[d] Novosibirsk State University, Novosibirsk, Russia

[e] Institute of Molecular and Cell Biology, Novosibirsk, Russia

[f] Scientific Research Institute of Complex Problems of Cardiovascular Diseases, Kemerovo, Russia

Abstract

Boron-neutron capture therapy (BNCT) is a unique form of adjuvant cancer therapy for various malignancies, including gliomas, primary malignant brain tumors, and especially glioblastoma, which is the most aggressive and rapidly growing glioma. Main advantage of BNCT is its ability to kill cancer cells selectively with minimum effect on healthy tissues. Previous clinical trials at nuclear reactors showed promising results in treatment of different malignancies, but for safety reasons the use of a nuclear reactor for BNCT turned to be impossible. Therefore, relatively safe accelerator-based neutron sources for BNCT are being developed by different research groups all over the world.

In Russian Federation, an original accelerator-based epithermal neutron source was constructed in the Budker Institute of Nuclear Physics in Novosibirsk Science City. The joint team of physicists, biologists and medical doctors carries out a series of experiments to assess the efficacy of the accelerator to be further used in preclinical and clinical trials. The results of those experiments are presented in this article. To determine optimal irradiation conditions and evaluate the effect BNCT on tumor cell survival, human glioma cells (U251MG) incubated in medium with boronophenylalanine (BPA) were irradiated in the phantom made of organic glass. Cells irradiated without boron were used as controls. Colony forming assay was used to estimate the viability and reproductive functions of cells and showed that generated neutron flux was effective to treat tumor cells with BPA and almost didn't influence cell viability without boron.

Additionally, the influence of neutron irradiation on SCID mice was evaluated depending on the dose of radiation and the dose of pre-injected sodium borocaptate (BSH). A novel method of boron drug delivery to the tumor tissue by means of pegylated liposomes with a fluorescent label is proposed. The method can increase the efficacy of BNTC and determine the localization of the compound in tissues.

Key words: BNCT, accelerator-based neutron source, glioma, colony forming assay, boron delivery agents.

Introduction

The most advanced technologies in neurosurgery, including radio and chemotherapy, are not able to significantly extend life of patients with glioblastoma, the most malignant primary brain tumor [Bush et al 2016]. One of the key points, that is particularly difficult to influence on, is the invasion of tumor cells into normal brain tissue, and no matter how well the brain surgery is performed, how ideally the adjuvant therapy is carries out, the median survival of patients with glioblastoma is limited to approximately 14.6 months after the diagnosis according to clinical trials [Stupp et al 2009] with 5-year survival ratio of 5% [Delgado-López and Corrales-García 2016]. To effectively control tumor growth, specific technique is required to impact single glioblastoma cells, which, in some cases, migrate over large distance from the primary tumor site causing further progression and poor prognosis [Able et al 2011, Baker et al 2014, Zaboronok et al 2014].

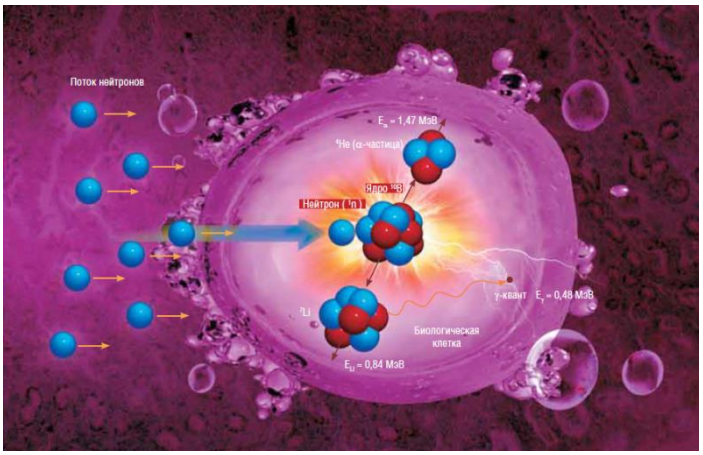

Fig.1 The energy of the nuclear reaction is emitted particularly in the cell that contains the boron-10 nucleus, which captures the neutron, leading to the destruction of the tumor cell. The use of boron-10 carriers, which are selectively accumulated in tumor cells, allows destructing tumor cells while sparing normal unchanged cells.

Boron neutron capture therapy (BNCT) is a unique type of radiation therapy carried out at a cellular level and directed first of all to the treatment of invasive tumors. Theoretical background for BNCT was first proposed by G. L. Locher in 1936 [Locher 1936], but practically the method was successfully utilized much later. The method is based on the interaction of two relatively harmless components: boron-10 nucleus and a thermal neutron. The treatment process is the following: a compound containing a stable isotope of boron-10 is administrated by the patient, the drug accumulates in tumor cells, irradiation of the tumor region with epithermal neutrons is carried out, the neutrons are captured by boron-10 atoms and the latter are converted to unstable boron-11 atoms, which decay into alpha-particles (helium nuclei) and lithium nuclei (Figure 1).
Along their pathway, which is limited by a single cell diameter, alpha particles and lithium nuclei transfer their energy of 2.31 MeV to tumor cell structures, including DNA. Thus, the efficacy is achieved by the influence on individual tumor cells located in the normal brain tissue while preserving the latter, since the path of the particle is less than the size of a single cell (around 7 to 5 microns). In BNCT, we initiate a nuclear reaction with the formation of alpha radiation at a cellular level, which is unique among other types of radiation therapy. Preclinical and clinical studies of BNCT were held in different countries around the world. In the beginning of the 21st century a group of researchers from the University of Tsukuba carried out a pilot clinical trial on

BNCT in patients with glioblastoma and demonstrated the efficacy of the method compared to the standard radiation therapy, showing up to about 2-fold increase in median overall survival in patients with confirmed glioblastoma, including one patient survived for over 9 years after initial BNCT [Yamamoto et al 2009, Matsuda et al 2009, 2011, Nakai et al 2011]. At that time, a nuclear research reactor JRR-4 in Tokai village was used as a neutron source. In spite of the promising results, the use of the nuclear reactor was frequently interrupted due to regular inspections for safety, and finally was prohibited after the accident at the Fukushima nuclear facility in 2011.

The safety concerns forced out research and 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 Russian Federation such an accelerator was constructed at the Budker Institute of Nuclear Physics in Novosibirsk Science City [Taskaev 2015]. The source of epithermal neutrons was developed on the base of a new type of proton accelerators – a tandem accelerator with a vacuum insulation [Bayanov et al 1998, Ivanov et al 2016] and a lithium target [Bayanov et al 2006], at the moment being a unique facility in the world to provide neutrons of desirable energy range for experiments. The accelerator is specially designed to be mounted in oncological centers and passed initial tests [Kanigin et al 2015, Iarullina et al 2015].

In the current study we aimed to evaluate the efficacy of the accelerator-based neuron source at the Budker Institute of Nuclear Physics in cell and animal experiments and weigh the possibility to utilize that technology in treatment of patients with gliomas and other malignant tumors.

Materials and Methods

Human glioma cell line

U251MG cells were purchased from the Institute of Cytology of the Russian Academy of Sciences, St.-Petersburg, Russian Federation, cultured in Iscove's modified Dulbecco's minimum essential medium (MEM) (SIGMA 17633 Iscove's DMEM with L-glutamine and 25mH HEPES, without sodium bicarbonate), supplemented with 10% fetal bovine serum (Thermo scientific HyClone SV30160.03 HyClone UK ltd.), maintained at 37˚C in 5% CO_2.

Fructose-BPA solution

p-boronophenylalanine (BPA) was purchased from KATCHEM Ltd. (Praha, Czech Republic). The enrichment of 10B 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 approximately 1100 μg of 10 B/mL [Yoshino et al 1989]. Fructose-BPA complex were added according to the previously evaluated boron accumulation in cell lines [Yoshida et al 2002].

Cytotoxicity assay

To determine reasonable and maximum non-toxic boron concentrations, the cytotoxicity evaluation was performed as described in our previous work (Zaboronok et al., 2013). 100 µl of MEM with $4x10^4$ of cells was placed in each well of 96-well plates and incubated for 24 h. The medium was replaced by MEM with BPA (0-320µg boron/ml) and further incubated for 24 h. MEM with boron was removed; the cells were washed with PBS. 2 ml of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution with PMS (Cell Titer 96® AQueous One Solution, Promega, USA) was mixed with 10 ml of MEM, 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 the Budker Institute of Nuclear Physics (Novosibirsk, Russian Federation). After 24-hour-incubation with BPA containing boron-10 (40 µg/ml) human glioma cells in the medium with BPA were placed in plastic vials in a phantom made of organic glass under the lithium target of the tandem accelerator with vacuum insulation (Figure 2). The irradiation was performed for 1-2 hours with the following accelerator settings: 2.0 MeV proton energy, 1-3 mA proton current. Epithermal neutron flux in the cells is equal to 10^8 1/(cm^2 s mA).

Fig.2 Irradiation experiments. U251MG cells 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) at the Budker Institute of Nuclear Physics, Novosibirsk Science City, Russian Federation..

Colony forming assay (CF-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 et al 2006, Zaboronok et al 2013]. In short, 2 weeks after the irradiation the dishes were washed with PBS, the cells were fixed with glutaraldehyde, stained with crystal violet, and dried. The colonies of 50 cells and over were counted for each sample and each irradiation dose. The results were compared to controls irradiated without boron.

SCID mice neutron irradiation

In the experiment, 14 immunodeficient SCID mice were used. Six of them were intraperitoneally injected sodium borocaptate (BSH) at a dose of 200 mg/kg (mice 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 BSH 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, what helps to decrease the irradiation dose to radiation sensitive areas of the animal body, which are normally shielded (Figure 3).

Fig.3 Animal positioning. Mice were placed radially in the container, with their heads inside.

The irradiation was performed 2 hours after the BSH injection. In every irradiation group with or without BSH injection, 2 mice were irradiated within 30 minutes, 2 mice – 60 minutes, and 2 mice – 90 minutes. Such time intervals are typical for BNCT. Between the irradiation sessions, the body temperature control was performed using a digital thermometer. Mean mice body temperature was around 27-29 ˚C. Irradiation doses received by mice organs did not exceed 5.7 Gy-Eq in mice with injected BSH and 2 Gy-Eq in mice without BSH injection. Such doses were accounted as tolerable, i.e. the exposure could not cause any incompatible with life radiation damage. After irradiation mice were transported to the designated area of the animal center for further follow up. The observation period lasted 1 month. To assess the effects of different doses of radiation on animal bodies after the observation period, the euthanasia was performed by craniocervical dislocation. Animal organs (kidney, liver, brain, heart, spleen) were dissected and fixed in in formaldehyde solution.

Results and discussion

BPA cytotoxicity

Tolerable concentration-dependent toxicity of BPA was observed against U251MG cells (Fig. 4). At the range of 0 to 320 µg/ml of boron, the lowest toxicity was observed within 10 to 40 µg/ml of boron, showing maximum proliferation rate of over 90%. The lowest proliferation rate of 0.844 ± 0.028 was relatively high and was observed at the maximum concentration of 320 µg/ml. The cytotoxicity assay proved the non-toxic nature of BPA. Thus, for the neutron source efficacy evaluation in this study we used well tolerated concentration of 40 µg/ml of BPA to minimize the effect of the drug on glioma cell colony formation.

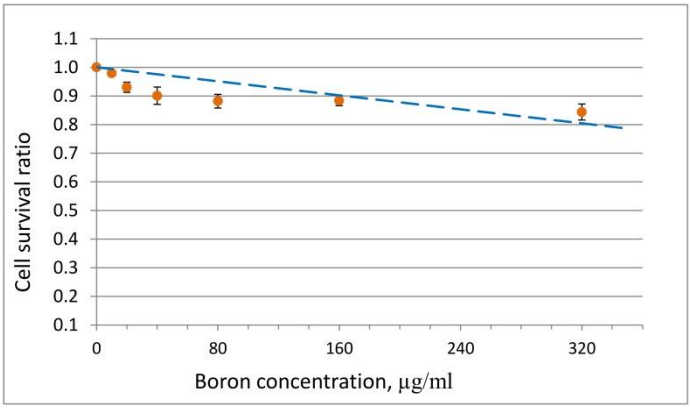

Fig.4. BPA cytotoxicity against human U251 malignant glioma cells. Cell survival ration depending on boron concentration (0-320 µg/ml). Data are represented as means±SDs.

Glioma cell survival after neutron irradiation with BPA

Colony forming abilities of U251 MG cells with BPA (40 µg/ml) and without the compound are presented in Figures 5 and 6. Neutron capture by BPA decreased tumor cell survival exponentially depending on the amount of delivered neutron radiation, showing the cell response to the therapy. The most significant effect was observed at the maximum amount of delivered neutrons. P-values were determined by one-way analysis of variances (ANOVA).

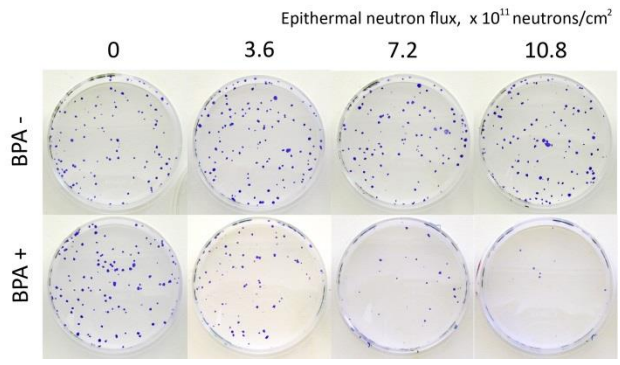

Fig.5. Colony forming abilities of U251 MG cells with and without BPA depending on epithermal neutron flux (3.6 – 10.8 x 10^{11} neutrons/cm^2).

Neutron irradiation provided efficient tumor cell proliferation control of U251MG cells, showing insignificant influence when applied without boron. The obtained data comply with previously reported data on the effect of nuclear reactor-based neutron source.

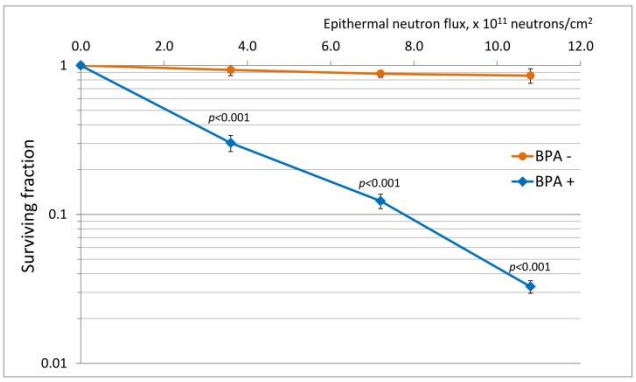

Fig.6. U251 MG cells survival ratio with and without BPA depending on neutron flux. Data are represented as means±SDs. P-values by one-way ANOVA.

Colony forming abilities of U251MG cells decreased exponentially and significantly differed from those of the cells irradiated without BPA. The minimum effect of neutrons without boron showed the safety of the accelerator-based neutron source in general to be used in preclinical in vitro experiments.

Neutron irradiation influence on laboratory animals

All mice were alive 1 month after the irradiation. External pathological signs of the irradiation were not detected. According to the results of histological examination of liver tissues in all animals exposed to radiation, variable moderate or significant focal and subtotal hydropic dystrophy without the correlation with the time of exposure and the injection of the boron-containing compound (Figure 7).

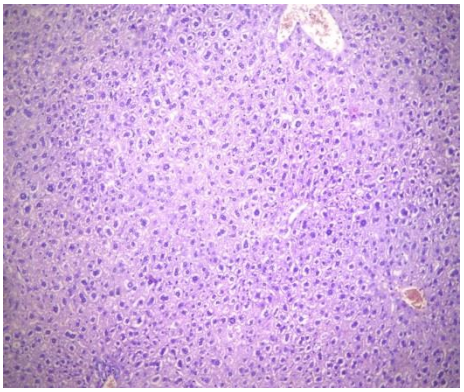

Fig.7 Most hepatocytes are in a state hydropic (protein) dystrophy with a large vacuolization and fine and eosinophilic granularity in the cytoplasm (x200).

Unlike in the irradiated animals, there were no significant morphological changes in liver biopsy tissue in the intact animals found (Figure 8).

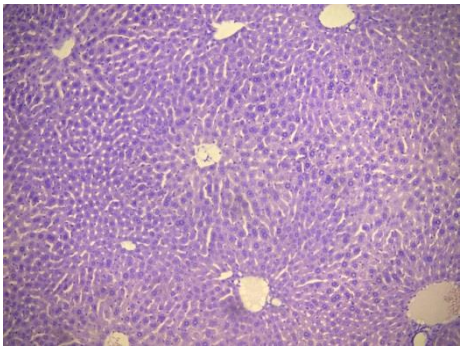

Fig.8 Hepatocytes with smooth clearer contours, regular shape, homogeneous cytoplasm. There are some hepatocytes with small eosinophilic granulosity (x100).

The morphological examination of the renal tissues of animals 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 the injection of the boron-containing compound (Figures 9, 10).

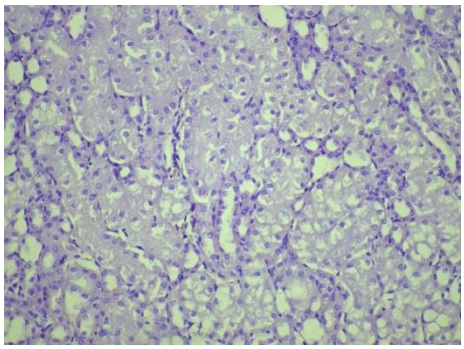

Fig.9 The epithelium of the convoluted tubules has got signs of hydropic distrophy: uneven grainy cytoplasm, vacuolization, and the blurred apical edge (x100).

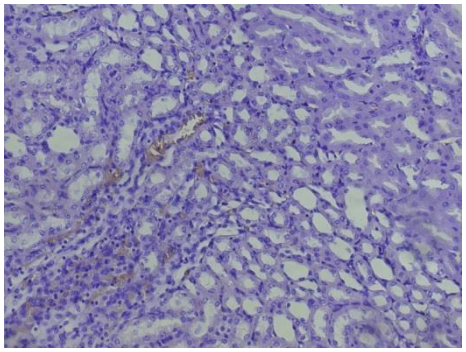

Fig.10 The epithelium of the distal tubules is without significant pathological changes. In the stroma in the area of the pyramids there are foci of minimum lymphoid infiltration (x100).

In contrast to irradiated animals, significant morphological changes in kidney tissues were not found in intact animals (Figure 11).

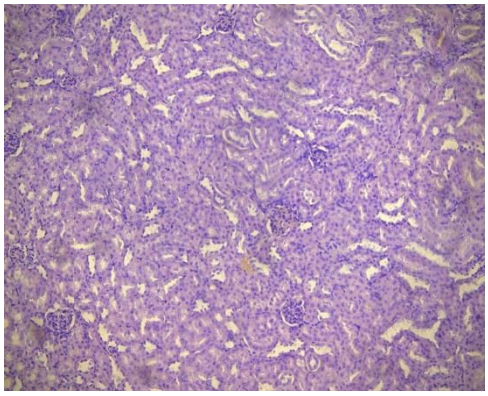

Fig.11. The division into cortex and medulla in kidney tissue is preserved. The glomeruli are lobed, the typical morphological structure is preserved. The epithelium of the convoluted and distal tubules is without significant pathological changes (x100).

Structural changes in the spleen of the tested animals are noticeable (Figure 12).

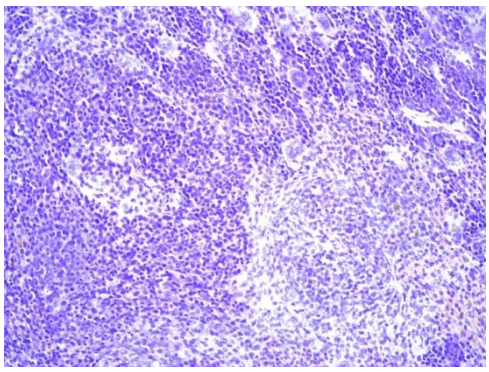

Fig.12 The division into red and white pulp is indistinct. The number of white pulp follicles is significantly decreased, the remaining follicles are reduced in size, the division into the mantle and marginal zones is absent. The red pulp contains blood sinuses, hemosiderin, small lymphoid cells, and a large number of loose-lying megakaryocytes (x100).

In the heart and brain tissues no significant morphological changes were found. According to the results of histological examination, we found the influence of neutron irradiation on liver and kidney tissues causing variable hydropic dystrophy, as well as on the spleen cells causing the decrease in white pulp follicles. Structural changes were not found in the brain tissues, which were mostly exposed to the neutron radiation. Thus, as a result of the animal experiment, we can conclude that the dose received by the healthy tissues of immunodeficient mice during irradiation can be accounted as tolerant and can be used in the following animal experiments with orthotopic tumor xenografts.

Future perspectives in preclinical and clinical application

Boron delivery agents

In the present study we used previously clinically approved BPA and BHA boron-containing agents. These drugs were successfully applied in BNCT for malignant tumors, including head and neck cancer [Barth et al 2012, Aihara et al 2014, Wang et al 2016], melanoma [Menéndez et al 2009], brain tumors, including gliomas [Yamamoto et al 2004, 2008, 2009, Matsumura et al 2009, Matsuda et al 2009, Nakai et al 2011, Barth et al 2012], etc. In regards to gliomas, the properties of BPA allow this compound penetrating blood-brain barrier; accumulate in active tumor cells without accumulation in resting cells with tumor/blood ratio of 2-4 after intravenous infusion (250-900 mg/kg). In contrast to BPA, BSH does not penetrate intact blood-brain barrier and is distributed via passive diffusion through tumor-related capillary, providing tumor/blood ratio of 0.5-2 after intravenous infusion (100 mg/kg). Both drugs are available commercially; though require special procedures for international transportation due to the presence of boron-10 isotope in them. One of the week points of these compounds remains their selectivity and small number of boron atoms, therefore effective components, per molecule. The selectivity of BPA can be only related to the increased accumulation of phenylalanine (PA) amino-acid in gliomas, which therefore accumulate BPA similarly to PA. BSH doesn't possess even those properties and completely relies on passive tumor tissue penetration, being unable to be selectively accumulated in migrated single tumor cells.

In our previous experiments we tested boron-hyaluronic acid (BHA) compound, a water-soluble polymer-based compound, produced by a solid-state synthesis from the mixture of hyaluronic acid (HA) powder and

borax [Zaboronok et al 2015]. The targeting of tumor cells was suggested to be released through CD44 receptors, which are widely presented in tumors, and especially in gliomas. The compound showed high stability in water solution containing a large number of boron atoms in a single molecule, though showed poor accumulation in C6 rat glioma model, showing the necessity for its further modification to be used in animal experiments.

Though BPA and BSH showed their efficacy in preclinical [Yoshida et al 2004, 2014] and clinical trials [Chanana et al 1999, Wittig et al 2002, Burian et al 2002, Capala et al 2003, Busse et al 2003, Diaz et al 2003, Yamamoto et al 2004, Kankaanranta et al 2004, Coderre et al 2004, Miyatake et al 2005, Yamamoto et al 2008, 2009], and new clinical trials protocols had been developed [Aiyama et al 2011], more selective compounds would be beneficial to provide more effective tumor cell killing and prolong overall survival in patients with cancer. We focused on development of a substance, which could be accumulated by tumor cells in sufficient concentration (the optimal tumor/normal tissue boron-10 ratio was targeted as 3:1) [Barth et al 2012]. We also faced with more serious challenge of penetrating the blood-brain barrier and highly infiltrative glioma cells with their molecular heterogeneity [Byvaltsev et al 2015].

To improve the efficiency of therapy, we have proposed a method of delivery of boron-containing agents in brain tumor cells by the pegylated liposomes with a fluorescent label, to estimate the localization of the compounds in the tissues [Taskaev et al 2016]. A potential benefit from the use of liposomes is due to their capability of high penetration into cells. Liposomes are able to contain both hydrophilic and amphiphilic hydrophobic substances, which allows to use them as carriers of various boron-containing compounds. Furthermore, such carrier type can be effectively used in the treatment of brain tumors, as liposomes allow transporting boron compounds through the blood-brain barrier. Polyethylene glycol, which is included in the liposome structure, limits their recognition and capture by the cells of the reticuloendothelial system [Nakamura et al 2009], resulting in the accumulation of liposomes in tumor tissue due to passive targeting effect. Encapsulation of boron-containing compounds into the liposomes can improve treatment efficacy and reduce the standard doses of boron drugs, decreasing treatment toxicity, as well as its cost.

There are plenty of methods for detecting the presence and amount of boron in the tumor, but the intracellular penetration of boron can only be detected using various labels. To confirm the tumor intracellular import of boron-containing drugs, fluorescent labels, included in liposomes, can be effectively used. Our 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. In such a way the possibility to control the delivery of boron-containing drugs inside tumor cells is provided, thereby increasing the accuracy of determining the location of drugs at a cellular level. It becomes possible to determine the quantitative ratio of the luminosity inside the cell and the extracellular space.

The proposed boron-containing drug delivery method is planned to be used at the stage of preclinical studies on BNCT to determine the optimum concentrations of boron compounds and to select the time interval of the maximum drug concentration in the tumor tissue for the most effective BNCT.

BNCT facility in Russian Federation

Perfect delivery of boron-10 compounds might be beneficial only if we obtain the effective accelerator-based neutron source. Along with the development of new boron-containing agents, the construction of a patient treatment-oriented accelerator [Aleynik et al., 2014; Sorokin and Taskaev, 2015] and placing it in the specialized center for BNCT is necessary for progressing in the field of oncology treatment giving hope to patients with cancer of poor prognosis and to their families.

Successful experiments at the accelerator built in the Budker Institute of Nuclear Physics, which shower the efficacy of the Russian neutron source for BNCT, defined the main direction of activities by Russian research groups to focus their scientific efforts and the Russian Government authorities to support financially the project, which can make Russian Federation the leading country for BNCT in the world.

Acknowledgments

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