Journal of Radiation Research

Radiobiological response of U251MG, CHO-K1 and V79 cell lines to accelerator-based neutron capture therapy

--Manuscript Draft--

Manuscript Number:	JRRS-D-17-00190
Article Type:	Regular Paper
Keywords:	boron neutron capture therapy; accelerator-based neutron source; lithium target; boric acid; in vitro efficacy evaluation
Manuscript Region of Origin:	JAPAN
Abstract:	In the current article, we provide in vitro efficacy evaluation of an accelerator-based neutron source, constructed at the Budker Institute of Nuclear Physics (Novosibirsk, Russian Federation), for boron neutron capture therapy, which is particularly effective in case of invasive cancers. CHO-K1, V79, and U251MG cells were incubated with boric acid in specific concentrations to create a verified intracellular boron uptake. Irradiation was then done with epithermal neutrons for 2-3 hours in a plexiglass phantom using 2.0 MeV proton energy and 1.5-3.0 mA proton current that resulted in a neutron fluence of 2.16x1012 cm-2. The survival curves of cells loaded with boron were normalized to those irradiated without boron to exclude the influence of fast neutron and gamma dose components and fit to the LQ model. Colony forming assays showed the following cell survival rates (means ± SDs): CHO-K1: 0.348 ± 0.069 (10 ppm), 0.058 ± 0.017 (20 ppm), 0.018 ± 0.005 (40 ppm); V79: 0.476 ± 0.160 (10 ppm), 0.346 ± 0.053 (20 ppm), 0.078 ± 0.015; and U251MG: 0.311 ± 0.061 (10 ppm), 0.0131 ± 0.022 (20 ppm), 0.020 ± 0.010 (40 ppm). The difference between treated cells and controls was significant in all cases ($P < 0.01$) and confirmed the neutron source and irradiation regimen were efficient for control over cell colony formation. We believe our study will serve as a model for ongoing in vitro experiments on neutron capture therapy to advance in this area for further development of accelerator-based BNCT into the clinical phase.
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ETHICS **Human Studies** 1.I confirm on behalf of all named authors that this paper complies with the current ethical standards of the Helsinki Declaration (1964, amended 2008, Seoul Korea http://www.wma.net) of the World Medical Association. 2.All persons gave their informed consent prior to inclusion in this study. 3.This paper includes a statement that the patient's written consent was obtained 4.Any information, including illustrations is anonymized as far as possible. 5.The design of the work in this paper has been approved by the local ethical committees, received IRB approval and/or complies with the current standards in the	N/A

country of origin. 6.This paper states the name of the authorizing body on the title page.	
ETHICS <u>Animal Experiments</u> 1.I confirm on behalf of all named authors that the animal experiments in this paper comply with the Principles of Laboratory Animal Care (NIH publication No. 85–23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, were followed, as well as specific national laws (e.g. the current version of the Japanese Law on the Protection of Animals). 2.A statement regarding IRB approval or animal ethics appears in the first paragraph of the material and methods section.	N/A

1. Covering Letter

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Professor Kenshi Komatsu,

Editor-in-Chief

Journal of Radiation Research

August 21st, 2017

Dear Dr. Komatsu,

On behalf of the authors, I would like to propose the manuscript "Radiobiological response of U251MG, CHO-K1 and V79 cell lines to accelerator-based neutron capture therapy" for publication in the **Journal of Radiation Research**.

In the current article, we evaluate the efficacy of an accelerator-based neutron source for future application to boron neutron capture therapy (BNCT) using U251MG, CHO-K1, and V79 cells incubated and irradiated in the medium with boric acid in different boron concentrations with further cell survival evaluation using a colony-forming assay. BNCT is an alternative and unique radiotherapy method which can provide selective tumor cell ablation within healthy tissues and is specially designated to treat invasive cancers. The efficacy of BNCT has been proven using nuclear reactors worldwide, though due to safety issues, as well as the accident in Fukushima, the world BNCT community turned towards development of accelerator-based neutron sources to replace nuclear reactors. The effect of epithermal neutron irradiation might vary depending on boron accumulation in tumor cells. To avoid uncertainties related to boron accumulation we used boric acid which can be uniformly distributed both in solution and intracellularly to assure steady boron concentrations during experiments. At this initial stage, we showed high efficacy of the accelerator-based neutron source for BNCT and are proceeding towards more complicated in vitro and in vivo experiments. We believe that our study will help other researchers in the advancement of accelerator-based BNCT into clinical phase trials.

The article has not been under review in any other journal and we hope that it will be found suitable for publication in the **Journal of Radiation Research.**

With kind regards,

Alexander Zaboronok, M.D., Ph.D.

Title: Radiobiological response of U251MG, CHO-K1 and V79 cell lines to accelerator-based neutron capture therapy

Short Running: Efficacy of accelerator-based BNCT: in vitro study

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Conflict of Interest: The authors report no conflicts of interest.

Total Page Number: 13

ABSTRACT

In the current article, we provide *in vitro* efficacy evaluation of an accelerator-based neutron source, constructed at the Budker Institute of Nuclear Physics (Novosibirsk, Russian Federation), for boron neutron capture therapy, which is particularly effective in case of invasive cancers. CHO-K1, V79, and U251MG cells were incubated with boric acid in specific concentrations to create a verified intracellular boron uptake. Irradiation was then done with epithermal neutrons for 2-3 hours in a plexiglass phantom using 2.0 MeV proton energy and 1.5-3.0 mA proton current that resulted in a neutron fluence of 2.16×10^{12} cm^{-2}. The survival curves of cells loaded with boron were normalized to those irradiated without boron to exclude the influence of fast neutron and gamma dose components and fit to the LQ model. Colony forming assays showed the following cell survival rates (means ± SDs): CHO-K1: 0.348 ± 0.069 (10 ppm), 0.058 ± 0.017 (20 ppm), 0.018 ± 0.005 (40 ppm); V79: 0.476 ± 0.160 (10 ppm), 0.346 ± 0.053 (20 ppm), 0.078 ± 0.015; and U251MG: 0.311 ± 0.061 (10 ppm), 0.0131 ± 0.022 (20 ppm), 0.020 ± 0.010 (40 ppm). The difference between treated cells and controls was significant in all cases ($P < 0.01$) and confirmed the neutron source and irradiation regimen were efficient for control over cell colony formation. We believe our study will serve as a model for ongoing *in vitro* experiments on neutron capture therapy to advance in this area for further development of accelerator-based BNCT into the clinical phase.

Keywords: boron neutron capture therapy, accelerator-based neutron source, lithium target, boric acid, *in vitro* efficacy evaluation.

INTRODUCTION

Boron neutron capture therapy (BNCT) is a unique radiotherapy method that provides selective tumor cell ablation within healthy tissues to treat invasive cancers. The efficacy of BNCT from a nuclear reactor neutron source has been confirmed for certain malignancies, including glioma [1] and malignant melanoma [2]. However, safety issues, as well as the negative publicity surrounding the Fukushima accident, turned the world BNCT community towards development of accelerator-based neutron sources to replace nuclear reactors in both trials and therapy.

Recently, several accelerators destined for hospital placement have been introduced [3]. For BNCT purposes, a proton accelerator with vacuum insulation and a lithium target have been developed at the Budker Institute of Nuclear Physics (BINP), Russian Academy of Sciences (Novosibirsk, Russian Federation) [4]. *In vitro* experiments using tumor and normal cells are typically carried out at the initial biological efficacy evaluation stage. It is within this stage that the main contrast to standard radiotherapy is seen as BNCT efficacy depends not only on the irradiation source, but also on the accumulation of a boron-containing agent whose concentration in tumor cells directly influences the treatment effect. Boric acid has been proven effective to achieve uniform boron concentrations both within the medium and the target cells by a previously reported radiobiological dosimetry study at the reactor-based neutron source [5].

Any proposed replacement for traditional reactor-based therapies requires seminal *in vitro* studies to establish optimum dosages and cellular effects. Therefore, in the current study, we evaluated the efficacy of our accelerator-based neutron source using CHO-K1, V79 and U251MG cells incubated and irradiated in a boric acid-containing medium at different boron concentrations (0, 10, 20, 40 ppm) with absorbed dose calculations and further cell survival evaluation using a colony-forming assay (CF-assay). This study is one of the initial steps of a project on synthesis and evaluation of complex boron/high-Z element compounds for absorbed dose estimation and tumor localization during accelerator-based BNCT.

MATERIALS AND METHODS

Cell lines

Chinese hamster ovary cells (CHO-K1), Chinese hamster lung fibroblasts (V79) and human glioma (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 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 maintained at 37˚C in 5% CO_2 atmosphere.

Boric acid application

In vitro experiments were performed at the Institute of Molecular and Cell Biology (Novosibirsk, Russian Federation). The cells were incubated for 2 hours in a medium containing boric acid (Sigma-Aldrich, Inc., St. Luis, MO, USA) in different concentrations (10, 20, 40 ppm of ^{10}B). The cells without boron were irradiated and used as controls. At the indicated timepoints, medium with boric acid was removed separately for each sample, the cells were washed with PBS, trypsinized (0.05% trypsin-EDTA, Nacalai Tesque, Inc., Kyoto, Japan), counted and placed in 2 ml plastic vials in the original medium (Figure 1A).

Neutron irradiation

The samples were placed in a phantom made of organic glass at the depth of 3 cm [6, 7] (Figure 1B) and irradiated in a tandem accelerator with vacuum insulation (Figure 2A) with the epithermal neutron beam under the lithium target (Figure 2B). The irradiation lasted 2-3 hours with the following accelerator settings: 2.0 MeV proton energy, 1.5-3.0 mA proton current (providing the epithermal neutron flux up to $3x10^8$ $cm^{-2}s^{-1}$). The settings were adjusted to produce epithermal neutrons eligible for phantom penetration with subsequent energy decrease to maximize neutron capture by boron in the samples. The necessary depth of the plexiglass in the phantom between the target and the cells was estimated using the Monte Carlo method and provided the maximum thermal neutron irradiation of the samples. The neutron flux was measured by a detector with a lithium-containing scintillator (GS20, Saint-Gobain Crystals, Hiram, OH, USA). Neutron fluence was measured by activation of the gold foil.

Colony forming assay

After the irradiation, the cells were counted, diluted, and seeded into 6 cm dishes for CF-assay. After 1-2 weeks, the dishes were washed with PBS (Phosphate Buffered Saline), fixed with glutaraldehyde, stained with crystal violet, and dried. Colonies of 50 cells or more were counted for each sample. Cell survival fractions were calculated according to a previously adapted protocol [8, 9]. The results were normalized to controls, which were irradiated without boric acid to smooth the influence of concomitant fast neurons and gamma-rays, and the statistical significance was evaluated using one-way analysis of variance (ANOVA).

Radiobiological parameters evaluation

The cell survival data were fit to the linear-quadratic (LQ-) model using the SOLVER add-on in the Microsoft Excel. As the issue of the absorbed dose evaluation in the accelerator-based BNCT remains controversial, we based on boron concentration to calculate radiobiological parameters. Using α' and β' values, the boron concentration needed to control 90% of cell growth, C_{10} (instead of D_{10}), 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 $\beta' = 0$) equaled $ln(SF)/\alpha'$, otherwise:

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

positive values of C were used.

RESULTS

Colony forming assay

Three types of cells were incubated in boric acid in four different boron concentrations, which resulted in analysis of five samples for each cell line in each experiment. The dosages chosen were evaluated as the most likely, realistic concentrations that could be reached in a therapeutic situation. Dishes with stained colonies of each cell line after irradiation with boric acid in different concentrations are shown in Figure 3. The number of colonies in all cell lines decreased with the increase of boron concentration with the maximum effect at 40 ppm. Cell survival rates (means ± SDs) are presented in

Table 1, and the survival curves are plotted in Figure 4. The difference between treated cells and controls was significant in all cases ($P < 0.01$). This data shows the dosage-dependent effect of boron on neutron beam effect and that physiologically-relevant concentrations can produce a therapeutic effect.

Radiobiological parameters

The calculated parameters are summarized in Table 2. In two of three cell lines (CHO-K1 and U251MG) $\beta' = 0$, reflecting the linear decrease in cell survival, typical for high-LET irradiation. In V79 cell line both α' and β' parameters were present, showing different response of the cell line to similar irradiation regimen. C_{10} values reflected the sensitivity of the cells loaded with boron to neutron irradiation. This indicates that BNCT therapy would therefore be useful for multiple types of treatments.

DISCUSSION

Recently, a surge in medical interest has seen the development of accelerator-based neutron sources all over the world to replace dangerous reactors [3]. In this regard, several types of neutron-producing targets have been introduced: solid ^{7}Li(p,n) [10], Be(p,n) [11], ^{9}Be(d,n) [12] and liquid ^{7}Li(p,n) [13]. With this in mind, a team of physicists at BINP has developed a compact tandem accelerator with vacuum insulation and a lithium target, which might have certain advantages over other materials in the number and spectrum of produced neutrons [4]. Initially, in 1998 Bayanov *et al*. reported on neutrons produced as a result of a threshold reaction involving ^{7}Li(p,n)^{7}Be after application of a proton beam of 2-2,5 MeV and 10 mA on a lithium target [14]. Further development of the lithium target and the results of the neutron spectrum analysis were reported [15]. The first experimental results of neutron production using a 2 MeV-proton tandem accelerator with vacuum insulation were shown in 2008 [16]. Bayanov *et al.* have reported on neutron generation experiments on a new 2 MeV tandem accelerator using a specially designed lithium target [17, 18]. It was only then that radiobiological experiments became available after stabilization of the proton current to 1.5-2 mA was achieved [19] the size of the accelerator was reduced to make it more compact [20]. It is within this framework of rapid development that we conducted our initial studies to prove the efficacy of accelerator-based neutron sources.

At present, initial radiobiological experiments on tumor cells to evaluate the efficacy of the neutron source at BINP were performed with L-p-boronophenylalanine (BPA) [21, 22, 23], a boron

agent. This compound was previously used as a ^{10}B compound in clinical trials in reactor-based BNCT along with disodium mercaptoundecahydrododecaborate (BSH) [24, 25]. However, the results of such *in vitro* experiments highlighted a critical point, namely, that cellular boron accumulation depends on transport mechanisms and can be influenced by different factors, creating the necessity to search for ways to optimize cellular boron levels to those needed for therapy [26]. Such variations in boron compound accumulation can significantly alter the results of the treatment and might need further trials to establish baseline values and reliably predict clinical outcomes.

Therefore, in this study we focused on using an agent which can uniformly be distributed in the cells and medium to assure an intracellular boron concentration that would enhance neutron therapy. Our cell survival data confirm the efficacy of the accelerator neutron source with the lithium target at BINP to produce a sufficient number of neutrons to initiate boron neutron capture reaction within and in proximity to the tumor cells. The cell survival rate in each cell line is inversely proportional to the boric acid concentration (Figure 4), showing that the number of neutrons was sufficient to control further tumor cell growth and these results are comparable to those obtained at the nuclear reactor using tumor cells incubated with boric acid by Yamamoto et al in 2003 [5]. In our experiments, the proton current was steadily ramped up to 3 mA to lessen the irradiation time and was kept relatively stable during irradiation.

In our experiments, radiobiological parameters were calculated based on boron concentration and reflected the character of the cell response to irradiation conditions. The cell survival curves were normalized to the data on cells irradiated without boron to remove fast neutron and gamma-ray components with the assumption that all detected neutrons were slowed down by the plexiglass to thermal ones at the sample level and passed through the cells once. Thus, in two cell lines (U251MG and CHO-K1), the survival curves were linear with $\beta' = 0$, reflecting the effect of a high-LET radiation effect, typical for BNCT. In V79 cell line, the presence of both α' and β' parameters could show the multicomponent irradiation effect or specific response to BNCT, and the character of the survival showed the cells were less susceptible to our treatment. Generally, it might be difficult to exclude the influence of all side factors, as, regardless of our assumption, some percentage of neutrons may keep higher energies while passing the samples, and even reaching room temperatures the neutrons can move

in different directions inside and in the proximity with samples. Thus, the measures taken for more correct radiobiological parameters evaluation can be among limitations of this study. The irradiation effect was obvious for the cells, though the provided epithermal neutron fluence still might be insufficient for clinical trials. In this regard, further improvement of the accelerator, including stabilization of an increased (up to 5 mA) proton current and development of a new lithium target and neutron beam shaping assembly [7] is in progress.

CONCLUSION

We carried out an initial evaluation of an accelerator-based neutron source for BNCT at BINP using boric acid to create verified intracellular boron concentrations that avoided compound accumulation variations. Such variation depends on boron uptake mechanisms and might differ in each cell line. We also proved the ability of the irradiation source to provide efficient control over cell proliferation after boron uptake. We believe that our study will bring more clarity to ongoing *in vitro* experiments on neutron capture therapy and help other researchers to advance accelerator-based BNCT into the clinical phase.

FUNDING

The work was supported by the grant from the Russian Science Foundation [Project No 14-32-00006], Budker Institute of Nuclear Physics SB RAS, Institute of Molecular and Cell Biology SB RAS and Novosibirsk State University, and also partly supported by a Grant-in-Aid for Scientific Research (B) [17K15797], from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

ACKNOWLEDGEMENTS

We would like to thank all the staff of the BNCT research laboratory for their technical assistance and accelerator operation. We also thank Dr. Hiroaki Kumada, Faculty of Medicine, University of Tsukuba, for his helpful suggestions in radiobiological parameters calculation.

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Figures and Legends

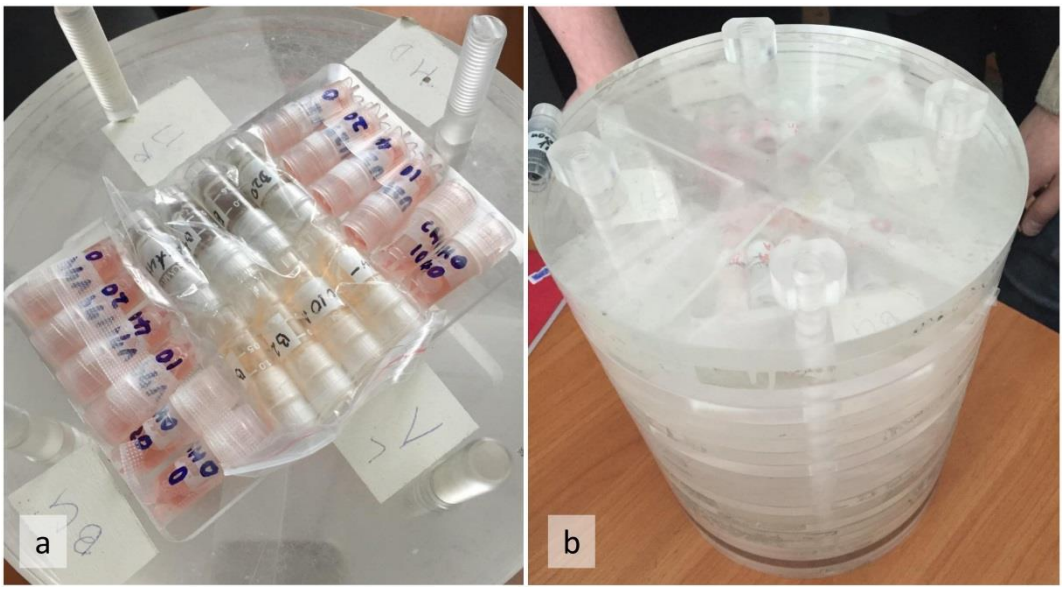

Figure 1. The samples in 2ml vials (a) placed in the modified Snyder head phantom (b).

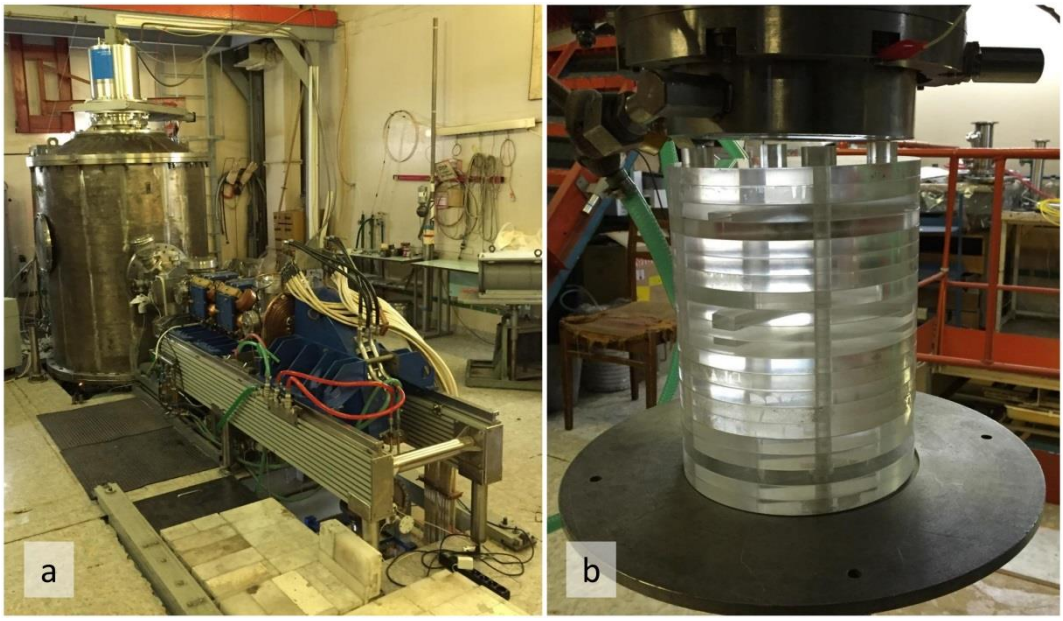

Figure 2. The accelerator-based neutron source at the Budker Institute of Nuclear Physics, Russian Academy of Sciences (a). Organic glass phantom set up under the lithium target (b).

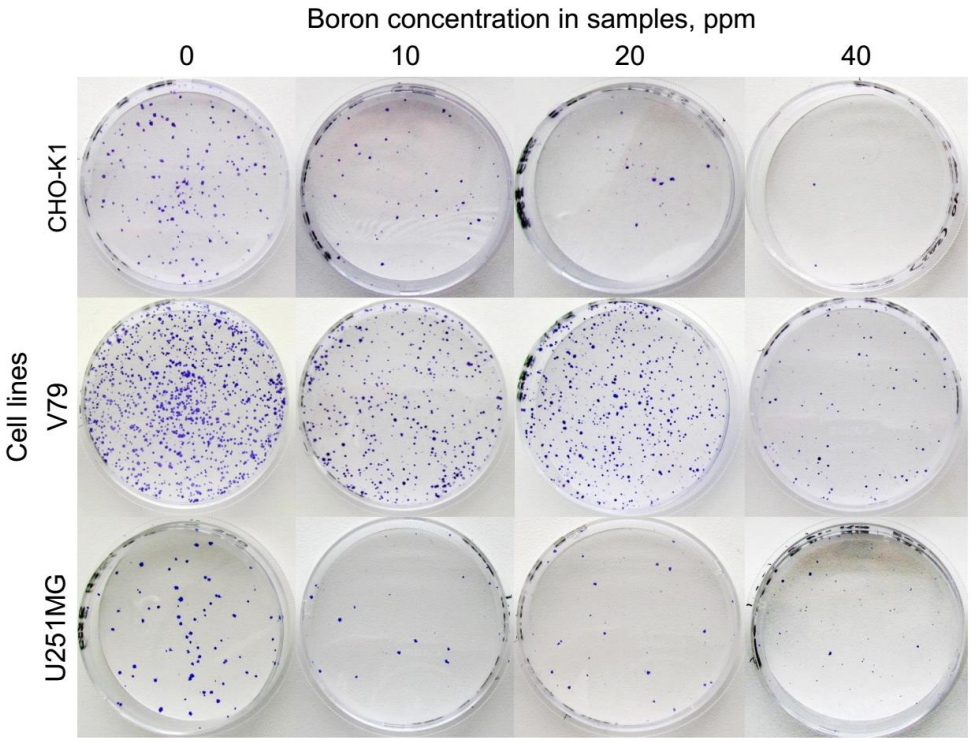

Figure 3. CF-assay results: 6-cm dishes with stained colonies of each cell line 1-2 weeks after neutron irradiation. CHO-K1 (upper), V79 (middle), and U-251MG (lower) cells. Boron concentration is 0, 10, 20, 40 ppm from left to right.

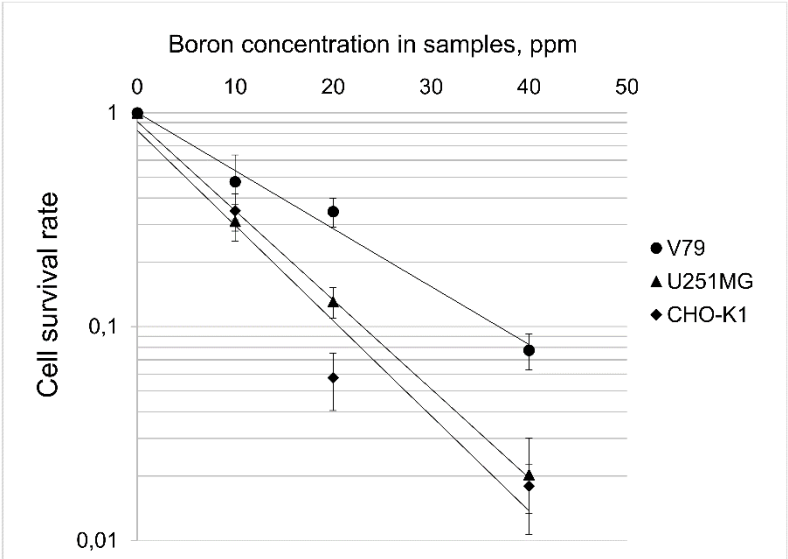

Figure 4. Cell survival curves depending on boron concentration in the samples. The data present means ± SDs. * $P < 0.01$ versus boron concentration of 0 ppm (one-way ANOVA).

Table 1. Surviving fractions of irradiated cells.

Boron concentration, ppm		10	20	40
SF	V79	0.476 ± 0.160	0.346 ± 0.053	0.078 ± 0.015
	U251MG	0.311 ± 0.061	0.0131 ± 0.022	0.020 ± 0.010
	CHO-K1	0.348 ± 0.069	0.058 ± 0.017	0.018 ± 0.005

Cell survival fractions (*SFs*) are presented as means ± SDs. All *SFs* significantly differed from controls ($P < 0.01$, ANOVA).

Table 2. Radiobiological parameters of irradiated cells.

Cell line	α'	β'	$C_{10,}$ *ppm*
V79	0,048008364	0,000390192	36,89722841
U251MG	0,102848442	0	22,38813781
CHO-K1	0,123231712	0	18,6850045

Parameters, such as α' and β' are presented as absolute numbers, C_{10} represents ^{10}B concentration needed to eliminate 90% of tumor cells, presented in *ppm*.