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Binary Radiotherapy of Melanoma – Russian Research Results

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1. Introduction

Radiotherapy (RT) in its different forms –distance, contact, intraoperative - currently is a powerful means of damaging cancer cells. RT is used both, as a radical monotherapy and in combination with different radiosensitizers or antitumor chemotherapeutic agents. The main problem of radiotherapy is the inevitable effect of ionizing radiation on normal tissues that results in unwanted side effects. Solving this problem was achieved practically by improvement of technical capacities of the ionizing radiation source and planning systems of irradiation procedure. The up-to-date therapeutic devices combine capacities of a medical imaging system (computed tomography) with a radiation therapeutic device. The combination according to the "two-in-one" principle ensures not only an ideal guiding of radiation on the target, but also constantly corrects the guiding, taking into consideration displacement of the target in the process of the patient's breathing, etc. The up-to-date planning systems help to increase reliability of precise guiding of ionizing radiation. Thus, unwanted action of ionizing radiation on normal tissues is reduced to the minimum, but is not excluded completely. In the last 100 years RT has transformed into a separate powerful trend in medicine. RT is equipped with sophisticated therapeutic devices widely used in oncological clinical practice - about 70% of oncological patients need these or those variants of RT. What are the prospects of the further improvement of RT? Obviously, the further technical improvement of therapeutic devices cannot be considered as a single way of RT development.

1.1 Binary radio therapy: What is it?

The most real trend of RT development is the development and introduction into clinical practice of Binary radiotherapy (BRT). Currently it is possible to speak about two types of BRT. They are neutron capture therapy (NCT), the idea of which was proposed in 1936 (Locher, 1936) and photon capture therapy (PCT). In English scientific literature is used the term "photon activation therapy". The principles of NCT and PCT (Khokhlov, 2004) are very similar. A malignant tumor is saturated with a preliminarily administered agent containing

an element which is highly capable of interacting with the particular external ionizing radiation. Then tumor distant irradiation is carried out and local release of energy directly in the target tumor takes place. The main task is to make the total dose in the tumor to be lethal for its cells, and radiation exposure of normal tissues not to exceed the limits of their radiotolerance. Ideally, this permits to replace sophisticated technical devices for guiding ionizing radiation into the tumor with self targeting system based on biological or pharmacokinetic principles, and to increase considerably specificity and therapeutic effectiveness of radiation treatment technology. It is this circumstance that is the main impetus initiating research in this direction. For implementation of BRT two components are needed - a source of ionizing radiation (flow of neutrons or photons of certain energy) and pharmaceutical which composition includes the elements being highly capable of interaction with ionizing radiation.

1.2 Physic principals of binary radio therapy

The basis of BRT are the physical processes occurring as a result of interaction of certain elements, being constituents of composition of special pharmaceuticals, with external ionizing radiation. Let us consider in more details peculiarities of BRT taking as an example boron neutron capture therapy (BNCT), gadolinium neutron capture therapy (GdNCT) and PCT.

^{10}B -Neutron Capture Therapy (BNCT) is based on nuclear reaction $^{10}\text{B}(n,\alpha,\gamma)^7\text{Li}$ occurs as the result of interaction between stable isotope ^{10}B with thermal neutron ($E_n=0.025$ eV). That interaction has very high probability ($\sigma=3890$ barn). Also as the result of the following nuclear reaction high energy short range charged particles – nucleus of helium (α -particle) and nucleus of lithium are emitted. These particles has high linear energy transfer (LET) to tissue (200 keV for α -particle and 350 keV for lithium nucleus) and short path length (about 14 μm) comparable to the diameter of a single cell. Such charged particles are equally lethal for oxygenated cells, hypoxic cells and cells in G0-stage. Sub lethal and potentially lethal damages caused by this kind of particles are nonreparable unlike the damages induced by the photon radiation. That is why BNCT is the most effective in treating tumors with cells are highly capable for DNA reparation. i.e. melanoma and glioblastoma. And if selective accumulation of ^{10}B in tumor cells is provided then selective radiation exposure could be achieved on the cell level: only tumor cells with ^{10}B inside would be destroyed leaving healthy tissues undamaged, thus killing all indefinitely small metastasis. In theory BNCT could overcome major limitations of photon radiotherapy: too high radioresistance of some tumor cells and to low radiotolerance of normal tissues.

For successful implementation of BNCT in clinical practice a complex of complicated chemical, medical, biological, physical and engineering problems must be solved. The most important task of them is development of ^{10}B -containing drug capable to deliver necessary therapeutical amount of ^{10}B into malignant tumor cells providing ^{10}B optimum intracellular distribution for a time necessary for neutron irradiation. It was calculated that for a fluence of 10^{13} n/cm² concentration of ^{10}B should be about 20-35 $\mu\text{g/g}$ or 10^9 atoms of ^{10}B per cell. To prevent damage of healthy tissues in the irradiated volume there must be at least 3 times less concentration of ^{10}B in them than in the tumor tissue. The requirement of the ^{10}B amount in the tumor cell depends greatly on it's intracellular localization. It's assumed that 2 $\mu\text{g/g}$ of ^{10}B is enough for successful BNCT in case of it's localization inside the cells nuclei. Therefore the radiobiological efficacy of $^{10}\text{B}(n,\alpha,\gamma)^7\text{Li}$ reaction greatly depends on so called "factor of compound" provided by peculiarities of chemical structure of boron with main substance, it's metabolism and it's distribution among the most important organelles of the tumor cell.

After many years of purposeful searches, two compounds were selected for BNCT - mercaptoundecahydrododecaborate (Sivaev, 2002) - $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ (BSH) and *p*-(dihydroxybor)-L-phenylalanine (BPA) (Snyder, 1958). As the clinical experience has shown, these compounds are not optimal for BNCT, but nevertheless it is possible to use BSH rather successfully at the combined treatment of brain tumors with BNCT (Japan, USA, countries of EU), and BPA - at the treatment of skin melanoma and its metastases (Japan, USA). Clinical trials are being conducted to study effectiveness of BNCT with BPA in treating brain tumors (Japan, USA). A parallel intensive scientifically grounded search of new, more perfect, boron-containing compounds for BNCT is going on.

An important aspect of the effective conduction of BNCT is also the existence of the methods which are capable to provide the optimal individual planning and control of its conduction. First of all, it is necessary to know the absolute concentration of ^{10}B , its microdistribution in the process of irradiation, that will allow to choose correctly the time of irradiation, to enhance accuracy of dosimetry and microdosimetry, thereby determining success of BNCT. In this connection of particular value is the development and mastering in clinical setting of the neutron radiation method of determining boron in tumor and normal tissues in real time by (n,γ) -reaction to ^{10}B , and also a number of other analytical methods allowing to determine intracellular concentration and location of boron, such as quantitative autoradiography of high performance, electron spectroscopy and others. Up to the present the evaluation of boron concentration in tumor at BNCT is conducted as a rule on the basis of preliminarily obtained data, for example, during surgical operation for removal of brain tumor, or indirectly - by the blood level of boron taking into account also preliminarily established ratio of its concentrations tumor/blood.

Interaction of photon radiation with different elements is characterized by dominating in a particular energy range (Fig. 1) of photon absorption by heavy elements (with $Z \geq 53$) on absorption by light elements composing biological tissues (H, O, N, C, Na, K, Cl etc.). This difference can be used for local increasing of energy absorption in the target (tumor) by administration or accumulating some heavy element in necessary region of irradiation.

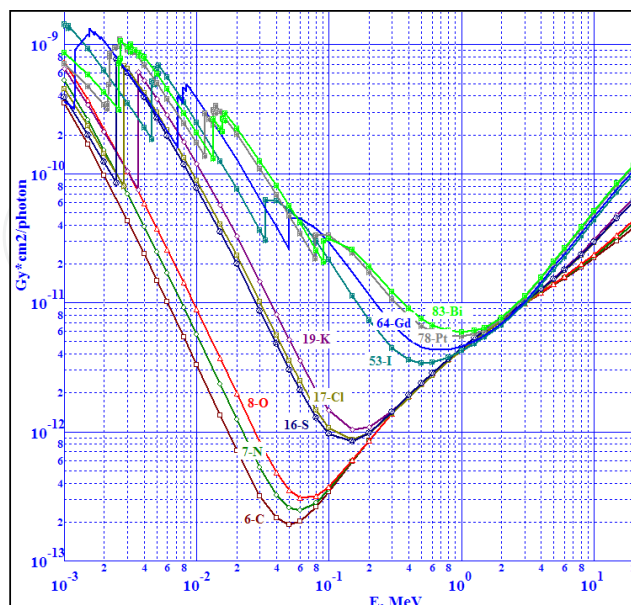


Fig. 1. Energy dependence of specific photon kerma for the basic elements of biological tissue and some heavy elements (Sheino, 2006).

In terms of Radiotherapy such approach can be implemented by administration or accumulation in a tumor volume the necessary amount of a pharmaceutical containing some heavy element with subsequent irradiation of the tumor region with X-rays of certain energy spectrum. As the result of such irradiation local increase of absorbed dose in the tumor occurs. In case of necessary amount of heavy element in the target is provided the increase of absorbed dose can be 2-3 times higher than for the same irradiation but with no drug administered (Karnas, 1999; Sheino, 2006). Emission of short range radiation caused by photoabsorption on heavy elements directly in the irradiated target is effective factor of tumor cell growth suppression. Significant that in PCT absorbed dose in healthy tissues could be lower than it's tolerant dose. Thus radiation influence on healthy tissues is decreasing and selectivity of tumor tissue damage is increasing during irradiation procedure. Such binary technology was called Photon Capture Therapy. Combination of biological self targeting of radiation and it's main localization in target volume makes PCT prospective Radiotherapy technology.

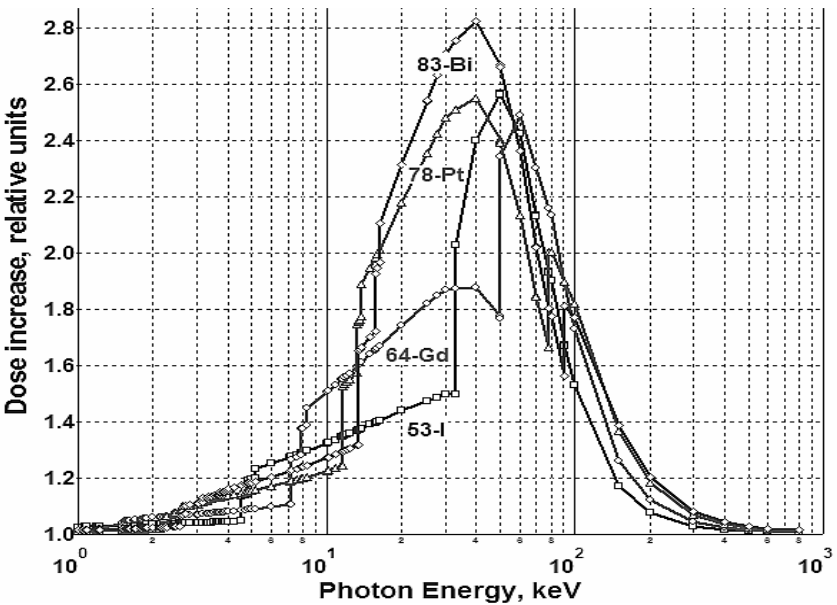


Fig. 2. Relative increase of a dose in a biological tissue for various elements with $Z > 53$ at their concentration of 1% in dependence on energy of photons. (Sheino, 2006).

Calculated estimation show (Sheino, 2006) that proper therapeutic efficacy of PCT could be achieved if the concentration of heavy element is around ~ 10 mg/g. At present there are no drugs with i.v. administration capable to accumulate in tumor tissues in such amount. That is why gadolinium containing MRI-contrast pharmaceutical Dipentast® (Russia) and intratumoral way of administration were used in our primary studies of PCT.

1.3 Tasks of research

The chapter presents the results of the preclinical studies on BRT conducted in Russia. Melanoma was chosen as the main object of the research. The studies were carried out in conformity with the effective RF requirements for three main directions: 1. the studies on the B-16 mouse melanoma cell culture; 2. the studies of mice with the transplanted B-16 melanoma; the studies on dogs with spontaneous B-16 melanoma. The similarity of canine and human melanomas permitted to replace the transplanted nude melanomas with the

model of canine spontaneous melanoma. The chapter presents the used neutron and photon bundles. It presents the main characteristics of the used pharmaceutical products with ^{10}B ($[^{10}\text{B}]$ -boron phenylalanine in the pharmaceutical form borate ether with D-fructose; $[^{10}\text{B}]$ -BSH - mercapto-closo-undecaborate as a sodium salt (Sivaev, 2002) and gadopentetat in the pharmaceutical form ensuring delayed elimination of the substance from the injection site). The results of remote consequences of BRT and traditional methods of melanoma treatment - surgical intervention, immune therapy, action of ionizing radiation (neutrons and gamma-radiation) are given. The comparison of therapeutic effectiveness of NCT with ^{10}B and Gd used as both monotherapy and in combination with adjuvant immune therapy with interleukin-2 (Roncoleukin[®]) is presented. Separately there are presented the results of the micropharmacokinetic studies - distribution of BSH and a number of new boron-containing agents across the main organelles of melanoma cell. For the results beyond the scope of the preclinical studies, the references to the published works are given.

2. Materials and methods

2.1 Irradiation of biological objects

Neutron irradiation was performed on the irradiation facilities of Moscow Engineering and Physics Institute research reactor IRT and research reactor RR-8 of RRC "Kurchatov Institute". The IRT facility includes the irradiation room for positioning cell cultures and laboratory animals including dogs in the neutron beam. The neutron beam delivered to the irradiation room has the following characteristics: thermal neutron flux - $1.1 \times 10^9 \text{ n/cm}^2\text{s}$, fast neutron flux - $5.8 \times 10^7 \text{ n/cm}^2\text{s}$, photon dose rate - $1.8 \times 10^{-4} \text{ Gy/s}$. Irradiation room is equipped with video surveillance to observe the state of irradiation object and with the physiological parameters monitoring system (heart and breath frequency, blood pressure, body temperature) as well as with drug delivery system. IR-8 reactor facility is designed for irradiation small object only - cell cultures and small animals. Thermal neutron flux on IR-8 facility was $1.2 \times 10^8 \text{ n/cm}^2\text{s}$, fast neutron flux - less than $0.96 \times 10^7 \text{ n/cm}^2\text{s}$, photon dose rate - $8.3 \times 10^{-7} \text{ Gy/s}$. Small animals were not anesthetized prior the irradiation. At the IR-8 animals were irradiated in Teflon[®] cages, at IRT animals were situated in lead boxes, which were limiting animal's movements but not interfering feeding and defecations. Local irradiation of transplanted into the rear paw tumor immobilized in advance was performed with dose of 2.5 Gy-Eq. Considering that poor fluence power thermal neutron irradiation was prolonged for time individual group as control for each animal group was used.

X-rays irradiations were performed using radiobiology autoprotective X-rays facility with anode voltage 220 kV and dose rate at the position of cell culture monolayer 2.0 Gy/min for cell culture irradiation and X-rays irradiation of mice bearing B-16 melanoma was performed with an X-ray unit with anode voltage of 150 kV and dose rate of 0.7 Gy/min at treated tumor volume.

2.2 Dosimetry

Thermal neutron absorbed dose at NCT was measured with prompt gamma neutron activation analysis (PGNAA). Average dose in tissue without drug was 0.25 Gy/h. Total absorbed dose was determined by 3 nuclear reactions, which provide the major part of absorbed dose (95-97%) during interaction of thermal neutrons with nuclides of biological tissue - $^1\text{H} (\text{n}, \gamma)^2\text{H}$; $^{14}\text{N} (\text{n}, \text{p})^{14}\text{C}$; $^{10}\text{B} (\text{n}, \alpha)^7\text{Li}$.

Dose rate of X-rays in irradiation position was performed using DKS-AT5350/1 dosimeter (Russia) and dosimetric films Gafchromic® EBT. Optical density of dosimetric films was measured on Varian Cary-50 spectrophotometer.

2.3 Animals and cell culture

B-16 melanoma cell culture for experiments was received from Blockin's Cancer Research Center (Russia) storage. These cells were grown as monolayer in glass flasks containing mixture of RPM6-1640 and Eagle's nutrient media with 10% calf serum and gentamicyne. Grown cells were suspended in saline. Cell concentration in suspension was 1000 per 1 ml.

Cultural flasks in PCT in vitro studies were divided to experimental and control groups. Both groups were irradiated using X-rays with 10 Gy, 20 Gy, 30 Gy and 40 Gy. Before irradiation Dipentast® was added into experimental flasks for 0.2 mg/ml Gd concentration.

Experiments in vivo were conducted on C57Bl/6 male and female mice bearing subcutaneously transplanted melanoma B-16. All mice were of veterinary certification.

The mice were subcutaneously inoculated into rear paw with $(3.5-4) \cdot 10^6$ melanoma cells. All NCT and PCT experiments were conducted 10-11 days after transplantation (tumor volume was about 0.9-1.0 cm³).

For NCT studying the mice were injected intraperitoneally with saline solutions of KUG-1 and BSH in amount of 0.2 ml containing 150 mg of boron per kg body weight for BSH and 25 mg of B per kg body weight for KUG-1. After injection of studied compounds (in 6 hours for BSH and in 24 hours for KUG-1) tumor was locally irradiated.

For PCT the animals were divided into four groups: 1- experimental group irradiated with X-rays and administered with Gd-DTPA before irradiation, 2 - mice only locally irradiated with X-rays but not injected with Gd-DTPA, 3 - nonirradiated mice but only administered with Gd-DTPA with the same amount that the experimental group, 4 - untreated mice. When tumor volume was achieved 1 cm³ 1 and 3 animal groups were intratumorally administrated with 0.175 ml Dipentast®. After administration mice from 1 group were immediately irradiated with X-rays in 20 Gy dose.

For NCT studies Dogs with spontaneous oral cavity melanoma were selected in LLC "Biocontrol" based on the results of clinical examination. The owners of the dogs were agreed on experimental treatment.

60 dogs with oral cavity melanoma were selected for the study. The dogs were examined before and after treatment using clinical and histological methods. The dogs were divided on the following groups: I group - without treatment; II group - surgical treatment; III - distant gamma-therapy, IV group - neutron therapy; V - BNCT; VI - GdNCT; VII - Complex treatment (NCT with adjuvant immunotherapy with Ronkoleukine®); VIII group - NCT without immunotherapy. BNCT was performed with [¹⁰B]-BPA in pharmaceutical form of boron ether solution with D-fructose. GdNCT was performed with Dipentast®.

In V group BPA were administered by two routes: in the artery which nourish tumor and intravenously (Fig. 3). Administration of the medicine was controlled by X-ray examination. Irradiation was performed 2.0-2.5 hours after BPA administration

2.4 Cell vitality assessment

For cell vitality assessment after irradiation MTT-test (Mosman, T., 1983) and clonogenic test were used. For MTT-test cells were sewed in 96-holes flat-bottomed plates and incubated during 3, 5, 7 days. 2-3 hours before termination 10 µl of (4,5-dimethylthiazoline-2)-2,5 diphenyltetrazoly bromide (final concentration was 1 mg/ml). After incubation cells were

centrifuged at 1200 rev/min during 7-10 min. Supernatant was carefully removed. 200 μ l of DMSO were added into each hole for formazane solution. Optical density of homogenous solution was determined in "Picon" photometer at 530 nm. Optical density of solution was demonstration of redox-intensivity in cell culture. For clonogenic test cells of both groups were sewed in 6-holes plates with full medium and in 3, 5, 7 days cell colonies were count using LOMO microscope.



Fig. 3. Preclinical trials of BNCT in dogs with spontaneous malignant melanoma. Infusion of BPA drugs using a feeding pump (left); X-ray control of the compound administration (right).

2.5 Quantification of boron and gadolinium

Boron tumor concentration was measured in vivo with PGNAA (Khokhlov, 2008; Khokhlov, 2009). During irradiation this concentration in tumor varied about 7-8 μ g/g for $[^{10}\text{B}]$ -BSH, 10-20 μ g/g for BPA and 10-11 μ g/g for KUG-1.

Gd tumor concentration varied about 10-100 mg/cm³, Gd concentration in biological samples was measured with neutron-activation analysis (Zaitsev, 2004 a) or using roentgen fluorescent analysis in X-Art-M concentration analyzer (Comita Ltd, Russia).

2.6 Subcellular boron distribution

In 5 min., 1 and 24 h after BSH, KUG-1 administration experimental tumors were excised, rinsed, weighed and homogenized. The cell organelles were prepared using routine techniques of differential centrifugation (De Duve, 1967).

Subcellular fractions (nuclei, mitochondria, lysosomes, cytosol) were received for further boron determination with PGNAA method.

2.7 Study of kinetics of B-16 melanoma cell population

Kinetics of B-16 melanoma cell population was studied at determination of melanocytes and DNA-synthesizing cells percentage in total studied tumor cells.

For this purpose tumors of exponential growth phase were used. On 7-th day after subcutaneous inoculation experimental C57BL/6 mice (body weight of 17-20 g; tumor size

of 4-5 mm in diameter) were intraperitoneally administrated with 100 mg of BSH per 1 kg of body weight. In 1 hour before euthanasia the mice were administrated with ^3H -thymidin (74 kBq/g of body weigh); as control administration saline was used. Excised tumors were used for preparation of cytological preparations. Melanocytes were determined as cells containing more than 5 granules of melanin (Alberts, 1989). Analysis of kinetics of DNA-synthesizing cells was studied in preparations covered with "M" emulsion counting ^3H -labelled melanocytes. These parameters were separately assessed for peripheral and central zones.

2.8 Study of cell distribution through the cell cycle

Alterations of the quantitative distribution through the cell cycle were also investigated. The distribution of tumor cells through the cell cycle was studied by flow DNA-cytofluorimetry on ICP-22 cytofluorimeter.

The BSH distribution was investigated in B-16 melanoma bearing male C57Bl/6 mice. The mice had a body weight (BW) of 17-20 g, the tumor was subcutaneously transplanted. BSH was administrated intraperitoneously. 2 dose groups were injected with 50 and 150 mg/kg body weight on 9-11-th day after subcutaneous tumor inoculation. In 3, 12 and 24 hours after administration tumors were excised (3 samples per time point). Tumor samples were used for preparation of cell nuclei suspension. This suspension was dyed with mixture of ethidium bromide and mitramycin (1:1).

2.9 Assessment of tumor growth

During experiments with transplanted tumors three reciprocally perpendicular tumor's diameters were daily measured and evaluated tumors volumes taking tumor's shape as ellipse:

$$V = (\pi/6) \cdot d_1 \cdot d_2 \cdot d_3 \quad (1)$$

Where V - tumor volume, d_1 , d_2 , d_3 - linear dimensions of ellipsoid, cm

For every temporary point tumor volume was normalized to it's volume at the beginning of exposure (V_0) and then V_1/V_0 against time from the beginning of exposure curves were constructed depending on every dose D and for every concrete mouse at adequate control for it ($D=0$). The parameters of tumor growth delay (T_d) and of time of tumor's volume duplication were evaluated from these curves as well as tumor growth index (2).

$$\text{TGI} = S_E / S_K \quad (2)$$

where: S_E - area under tumor growth curve of experimental group, S_K - area under tumor growth curve of experimental group.

Area under tumor growth curve was calculated as following:

$$S = \sum_{i=1}^{n-1} \frac{V_i + V_{i+1}}{2} t_i \quad (3)$$

where V_i - tumor volume at i^{th} measurement,

n - number of measurements,

t_i - period between nearby measurements.

2.10 Drugs

In our studies we have used the following medicines: [¹⁰B]-BPA (Khoknlov, 2001, Kulakov, 2001, Kulakov, 2002) – 4-(dihidroxyboron)-L-phenilalane (BPA, KatChem, Chehia), Katchem, (Czechia), chemical purity of 98 % , degree of enrichment of ¹⁰B – 99.7 %); borated ethers with D-fructose ([¹⁰B]-BPA-F) and D-galactose ([¹⁰B]-BPA-Gal); Na₂¹⁰B₁₂H₁₁SH - [¹⁰B]-BSH (Katchem, (Czechia) , chemical purity of 99 % ; degree of enrichment of ¹⁰B – 99.7 %; novel boron-contained compound KUG-1 (this is manufacturing code of compound), chemical purity of 99 % with natural boron and Dipentast® – Gd-DTPA based pharmaceutical with Gd content of 28.11% and semi-elimination period from site of injection - 55±6 min.). The dogs were intravenously administrated with boron-containing compounds while the mice were administrated with boron-containing compounds intraperitoneally. Dipentast® was administrated intratumorally at uniform distribution (4-6 injection per tumor) both in NCT and PCT studies.

3. Results of investigations

3.1 Cell pharmacokinetics

In the time interval from 1 to 12 h after the BSH administration relatively high boron content was in subcellular structures of tumor tissue. BSH was preferably accumulated in nuclei and mitochondria. (Table 1). These data suggest the BNCT with BSH might be more effective during time interval from 6 to 12 h after drug administration.

Subcellular fractions	Time after administration, h					
	1		6		12	
	Tumor	Liver	Tumor	Liver	Tumor	Liver
Nucleus	5.7	14	6.9	12.0	2.2	2.9
Mitochondria	3.0	6.1	2.6	5.1	2.1	0.5
Lysosomes	0.8	1.8	-	4.9	2.0	1.2
Cytosol	1.5	1.2	0.2	1.5	0.2	1.3

Table 1. ¹⁰B concentration in subcellular fractions of B-16 melanoma and liver in C57Bl/6 mice after BSH administration [¹⁰B μg/g]

In the time interval from 24 to 48 h after the KUG-1 administration maximal boron content was in nuclear fraction. This concentration exceeded necessary and sufficient concentration for successful NCT. Boron content in other subcellular fractions was sufficient for therapeutic efficacy of NCT. (Table 2). Therefore boron subcellular distribution had confirmed clinical perspectives of KUG-1.

Subcellular fractions	Time after administration, h			
	24		48	
	Tumor	Liver	Tumor	Liver
Nucleus	8.13	9.1	10.9	9.3
Mitochondria	4.59	3.17	4.29	4.56
Lysosomes	3.48	3.83	4.58	13.5
Cytosol	3.3	5.28	1.53	5.8

Table 2. ¹⁰B concentration in subcellular fractions of B-16 melanoma and liver in C57Bl/6 mice after KUG-1 administration [¹⁰B μg/g]

3.2 Influence on cell cycle and kinetics of cell population of B-16 melanoma

This research was devoted to study the influence of BSH – boron carrier at anticancer neutron capture therapy on cell population of murine melanoma B-16. It was proved that prevalent accumulation of tumor cells in synthetic and premitotic phases (S and G2/M) of cell cycle was caused by BSH effect. Probably this phenomenon of cell proliferation may be linked to compensatory population response on damaging factor of BSH (Ado, 1993). The detected activity of the latter coupled with its ability to penetrate into the nucleus and mitochondria may be the reason for its efficacy in NCT of melanoma and opens new perspectives of the well known drug. These data are shown in tables 3, 4.

Days after administration	Peripheria		Centre	
	Control	Experiment	Control	Experiment
1	4.0±0.9	10.9±1.1	9.2±1.3	12.0±1.2
2	13.1±1.2	15.3±1.3	16.7±1.3	8.8±1.0
3	22.0±1.5	18.9±1.3	17.6±1.4	12.3±1.2

Table 3. Quantity of melanocytes and labeled cells in peripheral and central zones of of B-16 melanoma after BSH administration (100 mg/kg of body weight)

Time after administration, h	BSH, ¹⁰ B μg /kg BW	Phase of cell cycle				
		IG ₁	IIG ₁	S	G ₂ /M	S+ G ₂ /M
3	50	19.5±2.0	58.4±5.7	13.7±1.4	8.4±0.9	22.1
	150	20.1±2.1	57.2±4.5	14.3±1.5	7.9±0.8	22
12	50	16.7±1.5	56.4±5.4	8.9±1.1	18.7±2.0	27.6
	150	16.2±1.3	54.6±5.1	16.6±1.3	12.7±1.3	29.3
24	50	17.0±1.8	57.9±5.6	10.1±1.1	15.9±1.6	25.1
	150	16.0±1.4	57.0±5.5	14.3±1.3	12.7±1.3	27.0
24	Control (saline)	20.1±1.9	59.2±5.7	14.0±1.5	6.7±0.7	20.7

Table 4. Influence of BSH on cell cycle of B-16 melanoma after BSH administration

So, BSH is the drug of threefold action and this action allows to realize full potential of BSH.

1. BSH is boron carrier to deliver boron into tumor and it’s vital structures.
2. BSH stimulates proliferating cells (S+G2/M), increasing sensitivity for all types of irradiation.
3. BSH influences on cell differentiation (synthesis of melanin is index of cell differentiation for melanoma).

The results of investigations demonstrated most efficacy of NCT during the first 24 h after BSH administration due to both subcellular distribution and influence on cell cycle. In further searching of new boron compounds for NCT it would be remembered that any chemical agent is probably active agent effecting on cell biology.

3.3 In vitro studies of photon capture therapy

On the base of obtained results of MTT-test and clonogenic test curves of dependence “ part of survived cells – exposition dose” were constructed. (Fig. 4 and Fig. 5).

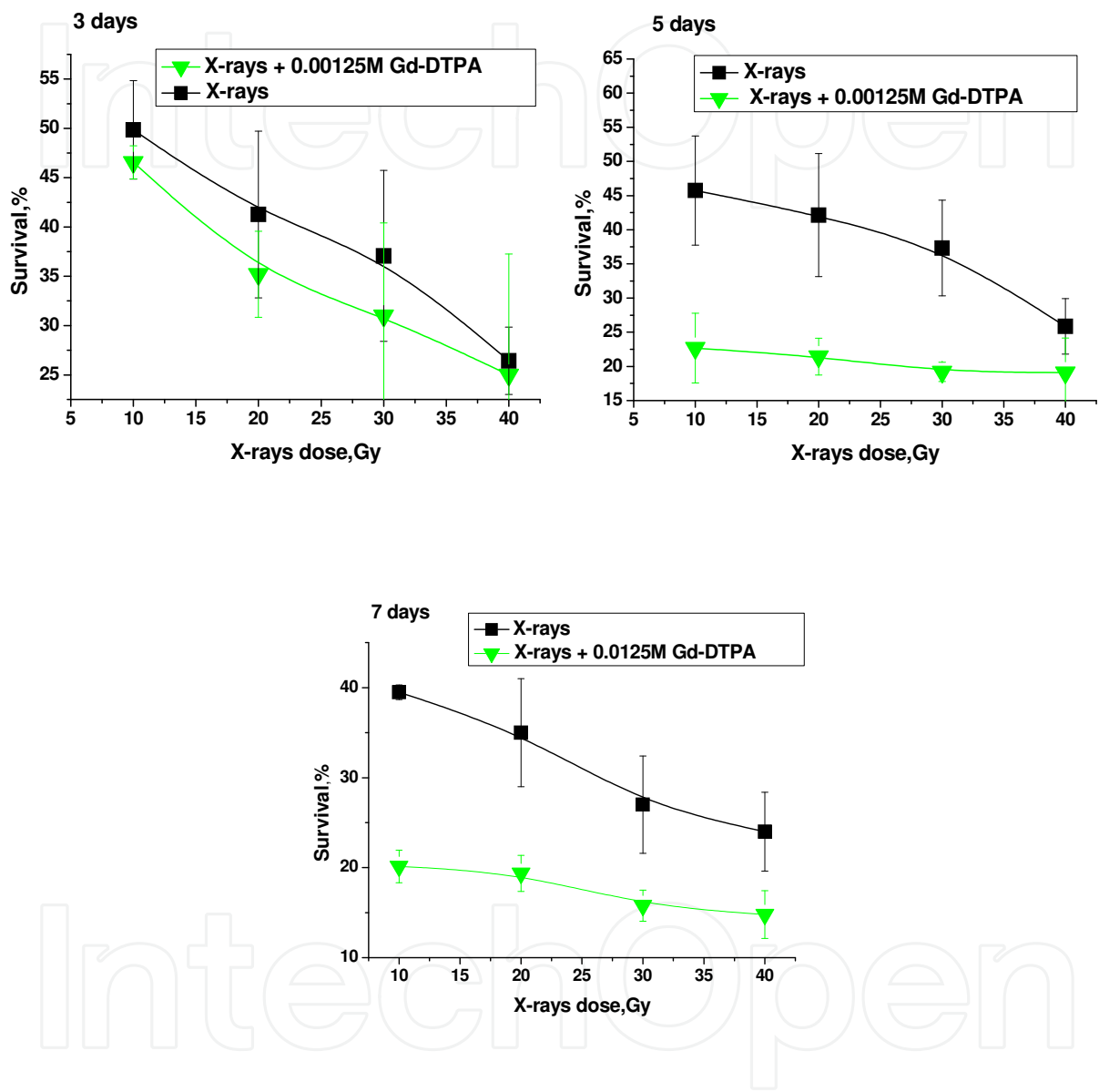


Fig. 4. Cell survival for control and experimental groups (MTT-test data) at 3rd, 5th and 7th days

MTT-test data had shown equal parts of survived cells in both groups on 3-rd day. On 5-th day part survived cells in experimental group was essentially low and was retained at this level to 7-th day. RGG-test data had shown (Fig. 5) practically complete suppression of cell growth in experimental group even at 10 Gy. In control group significant quantity of cell colonies were observed.

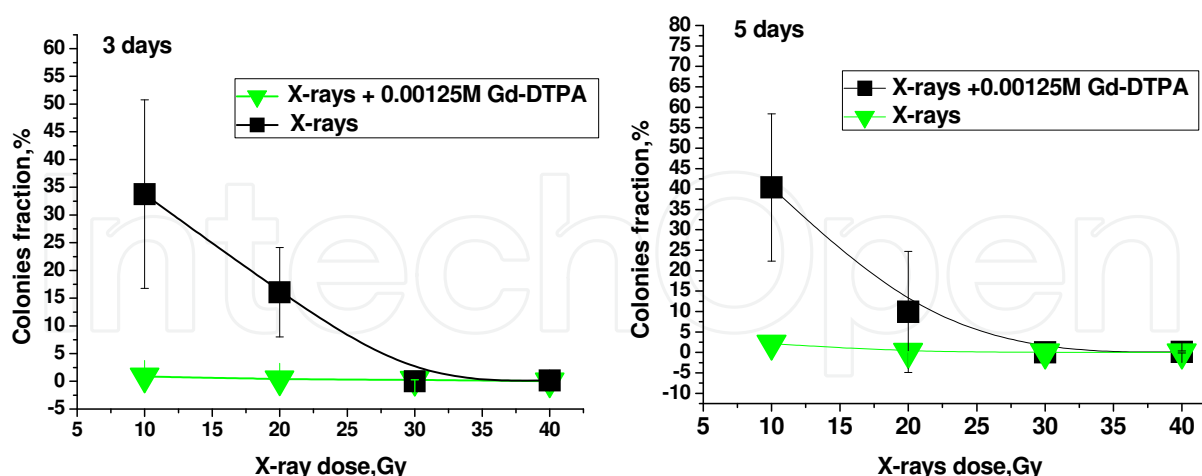


Fig. 5. Cell survival for control and experimental groups (clonogenic test data) , 3-rd and 5-th days.

3.4 Radiobiological studies in vivo

Studies of binary radio therapy in animals with potentially suitable for clinical application pharmaceuticals

3.4.1 Boron neutron capture therapy

The therapeutic efficacy of NCT was studied using two compounds: BSH and KUG-1 containing 55% and 20% boron relatively. PGNA data demonstrated that maximal boron content in tumor (12 $\mu\text{g/g}$ of tissue) was achieved in 1 hour after BSH injection decreasing by 6 hours. Then this level was not varying for 48 hours. On the same time the compound rapidly eliminated from all tissues adjacent to tumor excluding skin. As result in 12-48 hours interval there was sufficient for NCT ratio tumor/adjacent tissue including blood. The boron content suitable for NCT in the case of introduction of KUG-1 was accumulated in 24 hours and was constant not less then 15 hours. It should be noted that the total absorbed dose in tumor was determined from three major nuclear reactions - $(n,\gamma)^2\text{H}$; ^{14}N $(n,p)^{14}\text{C}$; ^{10}B $(n,\alpha)^7\text{Li}$ - contributing essential part in to absorbed dose at interactions between thermal neutrons and nuclides of living tissue and was equal to 95-97%. The time of tumor volume duplication (T_d) at NCT with BSH injection was increased to 14-15 days comparatively with 3-4 days for intact animals. In the case of Ph1 injection this time was prolonged to 8-10 days. The injection of Ph2 did not make this parameter differ from control meanings.

The increase of survival of treated mice was equal to 57% in the case of BSH administration and only 14% at KUG-1 administration in spite of KUG-1 accumulation coefficient was more than the same for BSH. It is caused with toxic side effect of KUG-1 (hepatic- and neurotoxicity).

KUG1 is perspective for purposes of NCT but needs a development for eliminations of toxic side effects. Results of irradiation are shown in table 5 and in figure 6.

B-10-carrier for NCT	KUG-1	BSH
Absorbed dose in tumor from reaction on B-10,Gy	1.76	1.23
Absorbed dose in tumor from reaction on N, Gy	0.42	0.79
Absorbed dose in tumor from reaction on H, Gy	0.45	0.36
Total absorbed dose in tumor	2.63	2.38
Concentration of B-10 in tumor, initial, µg/g	11.3	8.3
Concentration of B-10 in tumor, initial, µg/g	9.9	7

Table 5. Results of thermal neutron irradiation mice bearing B-16 melanoma

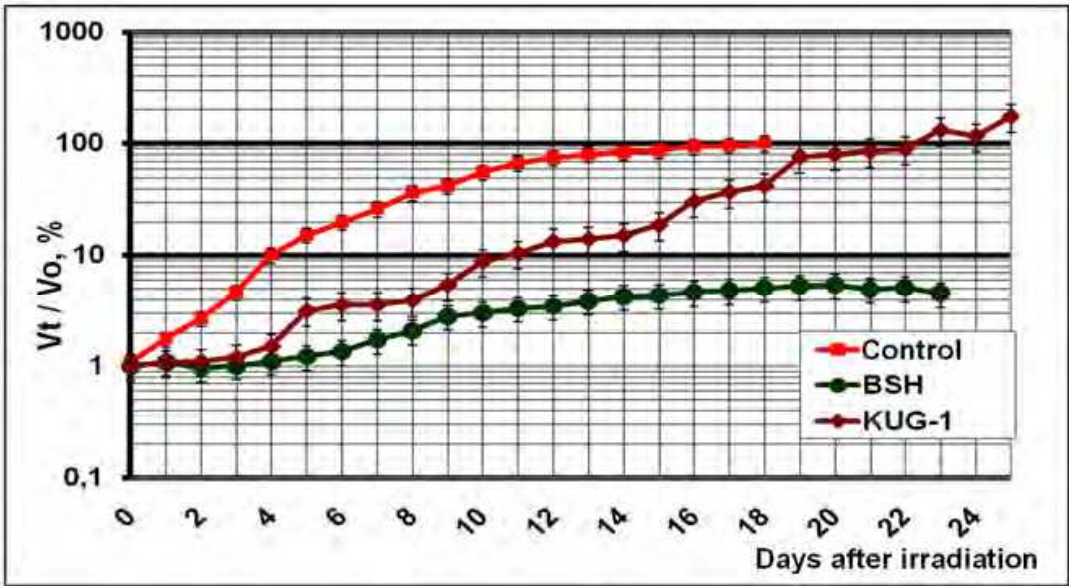


Fig. 6. Growth of transplanted B-16 melanoma in C57bl/6 after thermal neutron irradiation at presence of BSH and KUG-1

3.4.2 Photon capture therapy

On the base of obtained results of irradiation curves of tumor growth were constructed. (Fig. 7). Quantitative assessment of efficacy of each force was determined. (Table 6).

Criterion	Group 1	Group 2	Group 3
Tumor growth delay time, days	15±1	8±1	-
Tumor volume doubling time, days	16±2	11±1	-
Tumor growth index (IG)	0.1±0.03	0.25±0.05	0.75±0.05

Table 6. Quantitative parameters of effect assessment

Obtained data demonstrate that local irradiation of melanoma containing Dipentast® resulted in significant inhibition of tumor growth in comparison with irradiation only. Administration of Dipentast® without consecutive irradiation didn't result in expressed anticancer effect.

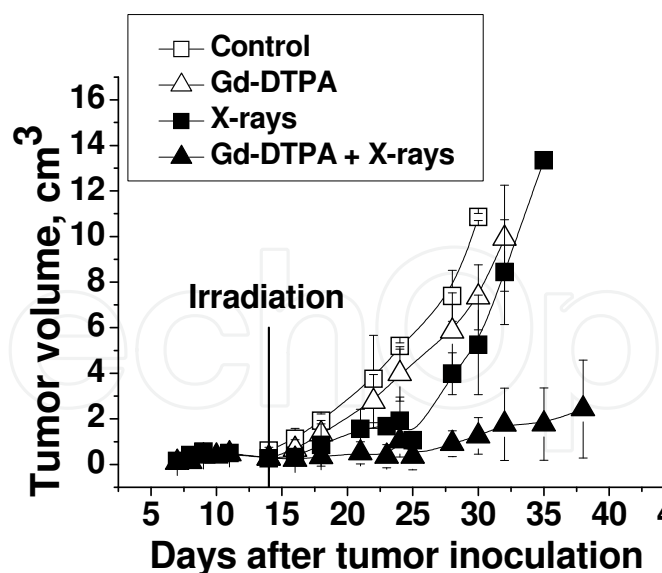


Fig. 7. Tumor volume growth kinetics for irradiated with Gd-DTPA injection group (▲), only irradiated group (■), only administered with Gd-DTPA group (△), untreated group (□)

3.5 Results of neutron capture therapy studies in dogs with spontaneous melanoma of the oral mucosa. Development of combined radiotherapy scheme on the basis of NCT and adjuvant immunotherapy

The improvement of the technology of neutron capture therapy under the condition of nuclear reactors, conduction of detailed studies with the purpose of comparative evaluation of effectiveness of different variants of neutron capture technology and traditional methods of treatment is an important and topical clinical task. The conduction of preclinical studies will permit to choose the most efficient scheme of treatment of melanoma, since melanoma of the oral mucosa in dogs may be regarded as a correct model of human melanoma.

The studies on effectiveness of BNC technology in treatment of spontaneous melanoma of canine oral mucosa have been conducted for several years. The preclinical study of neutron capture therapy on dogs with the diagnosis of oral mucosa melanoma has yielded the following results.

In group I of the animals which did not receive any treatment, the average animal life expectancy was 38 ± 13.6 days. 100% of the animals were euthanized due to their severe condition. The reason for euthanasia of such animals was local propagation of the tumor and systemic metastasis.

In the group of animals who underwent surgical removal of the oral mucosa tumor, 100% recurrence occurred within the period of 26 ± 12.5 days. In group III where the animals received traditional radiotherapy, stabilization of tumor growth was seen in 75% of cases, partial regression in 12.5% of animals and complete regression - also in 12.5% of animals. In 100% of cases resumption of tumor growth within 30 ± 5.5 days was observed.

Thus, both at surgical treatment and at radiotherapy recurrence occurred in 100% of cases. The statistical analysis showed that the duration of the recurrence-free period at the use of these methods of treatment was in fact equal.

This indicates that surgical treatment and radiotherapy are virtually low-effective methods of treating melanoma of oral mucosa in dogs at stage II-III of tumor process. This probably can be explained by the specific location of the tumor when it is impossible to perform

radical removal of the tumor. At these stages of tumor process the tumor size appears to be sufficiently large.

To evaluate effectiveness of neutron capture radiotherapy, that is, NCT, the results obtained were compared to the animals subject to the action of neutron bundle alone. In this group (IV, $n=5$) partial regression was attained in 80% of cases, and complete regression - in 20% of cases. In 100% of cases the continued growth of the tumor within 101 ± 28.8 days was also diagnosed.

In group V, where irradiation with neutron bundle was performed with a preliminary administration of boron agents, that is, BNCT, complete tumor regression was possible to be attained in 78% of cases. (Fig. 8) At BNCT, in cases of complete tumor regression no recurrence was observed. The absence of recurrence in complete tumor regression proves complete destruction of melanoma cells in the primary tumor focus.

In this study the boron product was administered by two routes: intravenously and regionally into the artery feeding the tumor. Having analyzed the results obtained, we got sufficiently significant data on that the route of the product administration exerts no effect on NCT effectiveness. Thus, during further studies of BNCT it is possible to administer ^{10}B BFA intravenously that facilitates conduction of the BNCT procedure.

During conduction of NCT with Dipentast®, that is, GdNCT (group VI), somewhat other results were obtained as compared to BNCT. First, tumor regression took place more slowly in at GdNCT comparing to BNCT, and complete tumor regression was noted only in 46% of cases (Fig.9). In 66.7% of animals recurrence of the tumor was seen 106 ± 7.5 days after the procedure of GdNCT. Nevertheless, the effect of irradiation at GdNCT was sufficiently high that suggested a possibility of development of an effective technology of melanoma treatment on the basis of GdNCT.

Since so far a small number of studies on GdNCT was conducted and there are no data on the optimal concentration of gadolinium inside the tumor which is necessary to achieve the tumoricide dose, we studied the GdNCT effect at different doses of gadolinium in tumor. The studied range of concentrations was from 10 mg/cm^3 to 100 mg/cm^3 . During the clinical trial the regularity was determined at which the best clinical effect of GdNCT was observed at the gadolinium concentration in tumor of up to 12 mg/cm^3 .

We also conducted the study on investigating distribution of the power of total dose depending on the gadolinium concentration inside tumor. Such study was performed on the basis of the developed model of GdNCT (1).

The study of total dose rate distribution during GdNCT at different concentration showed that at the gadolinium concentration over 12 mg/cm^3 the total dose in the tissue sharply decreases by several times. So, at concentrations of $6\text{-}12 \text{ mg/cm}^3$ the total dose power at the depth of 0.5 cm of tissue is $150\text{-}200 \text{ cGy/min}$ and does not increase with the growth of the gadolinium concentration in target (Fig. 10). Such effect is due to the fact that isotopes of gadolinium ^{157}Gd (the content in the natural element 15.68%) have high sections of capture of thermal neutrons. That causes an increase of absorption of thermal neutrons during GdNCT at high concentrations of gadolinium products. The so-called "shielding effect" occurs.

Thus, the analysis of distribution of the total dose, by using the solving of the equation of transfer of neutrons and photons by the method of discrete ordinates according to the RADUGA-5 program, showed that the optimal concentration of ^{157}Gd is within the range of $6\text{-}12 \text{ mg}$ of Gd in 1 cm^3 of tumor. These conclusions help to optimize subsequently the process of GdNCT for achieving the maximum result.



Fig. 8. BNCT Results of spontaneous melanoma of the oral mucosa: A - Melanoma before BNCT, B - 2 weeks after procedure BNCT. Full Tumor regression



Fig. 9. GdNCT Full regression of melanoma. A – Oral Melanoma, B - 1,5 months after GdNCT

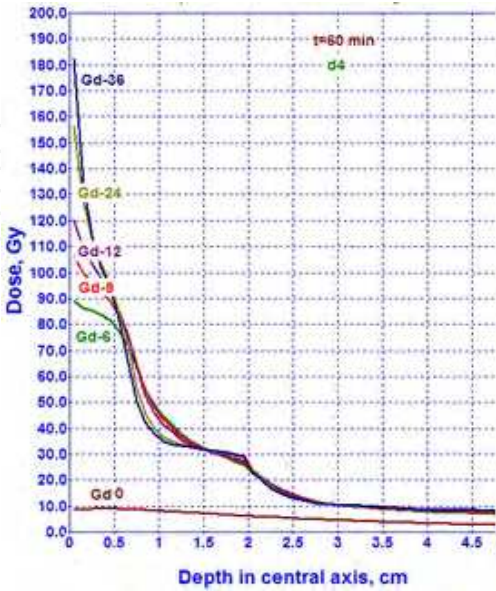


Fig. 10. Tumor dose distribution in different ¹⁵⁷Gd concentration.

Thus, the clinical results agree entirely with the further studies on investigating distribution of the total dose power depending on the gadolinium concentration inside tumor.

Comparing the time of recurrence in control and at GdNCT we found that the time of recurrence at GdNCT (106 ± 7.5 days) significantly exceeds the time of recurrence at surgery (26 ± 12.5 days) and at traditional gamma-therapy (30 ± 5.5 days). Such advantage of the GdNCT method comparing to traditional therapy is explained by a high local dose of irradiation of 80-100 Gy per the irradiated target.

The differences in the results of BNCT and GdNCT, in our opinion, may be explained by two reasons:

the differences in the pharmacokinetic properties of the used products - ^{10}B -boron phenylalanine during BNCT and the complex of gadolinium with DTPA during GNCT;

different products of nuclear and physical reactions of nuclei of ^{10}B and Gd with thermal neutrons.

During BNCT there is a selective accumulation of ^{10}B -boron phenylalanine inside the tumor cells. The ratio of ^{10}B concentrations in the tumor and in normal tissues is not less than 2.5. The run of alpha-particles which are formed during irradiation is commensurable with the cell diameter, and the nuclei of ^6Li – less than the cell diameter. Therefore secondary radiation affects predominantly tumor cells, by which a high effect of BNCT effect is achieved. And in the given study during BNCT we observed degree 1-2 of local radiation reactions.

During GdNCT the used product with gadolinium - Dipentast® is mainly in the intercellular space, and accumulation of the product inside tumor is achieved only by intratumoral administration of the product. As a result of absorption of thermal neutrons by the nuclei of ^{157}Gd there are formed high-energy gamma-quanta, low-energy electrons and roentgen radiation. It is secondary radiation that causes death of tumor cells. However, the character of secondary radiation leads to affection of healthy tissues as well. At the use of GdNCT degree 2 of local radiation reactions was diagnosed in all animals, and in one case - stage 4. Such difference in the mechanism clinically should manifest itself in an increase of side effects on healthy tissues during GdNCT. Indeed, during GdNCT the degree of radiation lesions of healthy tissues was somewhat higher (degrees 2 and 4 of local radiation reactions) than at irradiation with neutrons and during BNCT (degree 1-2 of local radiation reactions).

An increase of recurrence rate at GdNCT even in complete regression of tumor shows that at GdNCT it is not always possible to achieve death of all tumor cells. One of the reasons of this result may be the excessive concentration of ^{157}Gd inside tumor. As natural gadolinium has the section of neutron capture two orders higher than boron has, at its excessive concentration in tumor the effect of screening on the tumor surface may occur that does not allow to achieve destruction of cells in a deeper layer. During further studies, at improvement of GNCT technology, it is necessary to administer gadolinium product in such way so that the gadolinium concentration in tumor tissue would not exceed 12 mg/cm^3 .

Despite the conducted treatment, in all animals there was recorded systemic metastasis into the lungs, that is, despite local cure of oral mucosa melanoma, the further generalization of tumor process could not be avoided. Thus, NCT is effective comparing to other methods for local treatment of the primary tumor focus but influences in no way the process of formation of metastases. Therefore the task of this study was not only to evaluate NCT effectiveness during treatment of the primary tumor focus, also development of the

multimodality treatment aimed at both the primary tumor focus and prevention of quick metastasis of tumor. We formed two groups of animals - groups VII and VIII in which we determined life expectancy. These groups embraced the animals at one stage of tumor process in which complete regression was achieved as a result of NCT. In this study the adjuvant therapy influence on life expectancy of dogs with oral cavity melanoma (group VII) was investigated by using Ronkoleukin®. For inclusion into the scheme of treatment of melanoma on the basis of BNCT and GdNCT as the means hampering the process of metastasis, the Russian agent Roncoleukin® was chosen which is an immunostimulator, it enhances the antibacterial, antiviral, antifungal and antitumor immune response. We managed to carry out the study on a small group of animals, but the results obtained show significantly that life expectancy at conduction of immunotherapy increase on the average by three times. So, in the study group of the animals which received successful BNCT at stage II of tumor process and also receive adjuvant immunotherapy, the average life expectancy was about 305 days. In group VIII where BNCT was conducted without immunotherapy, the average life expectancy was 113 days.

Thus, in cases of successful NCT at concomitant conduction of immune therapy we observed the maximum life expectancies, and most such animals were long-living ones.

The studies performed by us have shown that treatment of oral mucosa melanoma should be of multimodality character and be aimed at both combating the primary tumor focus and remote metastases.

During conduction of NCT it is necessary to attain complete regression of tumor, it is in these cases that the maximum life expectancies may be reached.

This approach to the diseases, namely the combination of NCT and adjuvant immune therapy permitted us to gain the maximum results in treating melanoma.

In this chapter the results of complex method of oral canine melanoma are shown, but also we studied the possibility of NCT combination with other methods for canine osteosarcoma treatment. Induction chemotherapy, intraarterious administration of BPhA, bone resection, extracorporal BNCT of bone fragment, radiated bone implantation and adjuvant chemotherapy were included in treatment scheme (Fig. 11). 24 hours after operation dog can lean on the leg. 2.5 months after operation, results of biopsy showed absence of tumor cells in replanted bone. X-ray imaging showed full consolidation of replanted bone with maternal bone, without carcinogenesis signs (Fig. 11, 12), Total life span of the dog was 2.5 years without recurrence.

Results of highly malignant tumors treatment show that, instead of high metastatic activity and low traditional methods efficacy, it is possible to achieve high therapeutic efficacy with application of complex treatment scheme on the basis of NCT.

4. Conclusion

There is a large world positive experience of BNCT clinical application in combined treatment of more than 300 patients with brain tumors (Japan, USA, EU countries) and more than 30 patients with melanoma, including metastatic melanoma (Japan, USA), which shows the prospects for future development and optimization of this type of binary therapy. According to Japanese clinical data for 1968-1996, average life span of patients with brain tumors was from 640 till 1811 days, depending on histological type of tumor, without strong mental disorders. 6 patients after BNCT live more than 10 years. Average life span of such



Fig. 11. BNCT of primary osteosarcoma IIb stage. A, B – Roentgenoscopy before operation; C, D - Roentgenoscopy 2.5 months after surgery.



Fig. 12. A dog on walk 2.5 months after BNCT. Surgery region is marked in yellow

patients after traditional methods of treatments was 8-10 months. Doctor Nakagawa Y., taking into consideration BNCT opportunities and analyzing his own clinical experience, supposes, that BNCT is the best method for malignant brain tumors treatment, which needs further development of neutron beams and ^{10}B -containing drugs (Nakagawa & Hatanaka, 1997; Nakagawa et al., 2003). Preliminary analysis of melanoma treatment clinical results shows, that more than 2 years life span for patients with melanoma was 78%, treatment can be succeed in $T_{3-4} N_0 M_0$ melanoma in more than 90% of patients. (Mishima 1996; Busse et al., 1997; Barth et al., 2005).

Achieved by Russian scientists results prove the advantages of BRT in comparison with traditional methods of treatment. BPA is more effective substance for BNCT than BSH and KUG-1. Full tumor regression was reached in 80% of cases with application of BNCT, and in the group of GdBNCT full tumor regressions was in 50% of dogs with oral cavity melanoma, while the application of traditional gamma-therapy provides only 12,5 % of full tumor regression. Complex therapy, which combines BNCT with adjuvant immunotherapy with Ronkoleukin® (Interleukin-2) can increase the total life span more than 3 times. To compare with human life span we can speak about possibility of 5-10 years recurrence-free period for humans. As canine melanoma is the correct model of human melanoma, such complex method of treatment significantly more effective than traditional methods of treatment One of the reason of BNCT efficacy with BSH is the capability of BSH to penetrate into the cell nucleus and mitochondrions. This fact opens new prospects for future application of BSH in

BNCT. Complex treatment scheme, which combines traditional methods of treatment with BRT can significantly improve malignancy treatment.

Therapeutic possibilities of BRT are not finally defined, but this method of treatment has low cost and it can be easily installed and transported to other hospital. It makes us to intensify the studies for effective BRT technologies creation, putting more attention for creation of specific pharmaceuticals, which can accumulate in the tumor with tumor/normal concentration gradient more than 3. To minimize resources for new pharmaceuticals selection for further studies we apply developed screening scheme.

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