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Assessing functional suitability of a lyophilized formulation containing designed ankyrin repeat proteins for radionuclide imaging of HER2/neu overexpression in malignant tumors

Varvashenya R.N.[1, 2], Prach A.A.[2], Plotnikov E.V.[2], Deev S.M.[2, 3], Belousov M.V.[1, 2], Larkina M.S.[1, 2], Chernov V.I.[2, 4]

[1] Science and Education Laboratory for Chemical and Pharmaceutical Research, Siberian State Medical University 2, Moscow Trakt, 634050, Russian Federation

[2] National Research Tomsk Polytechnic University 30, Lenina Av., Tomsk, 634050, Russian Federation

[3] Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences 16/10, Miklukho – Maklaya Str., Moscow, 117997, Russian Federation

[4] Cancer Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Science 5, Kooperativny Str., Tomsk, 634009, Russian Federation

ABSTRACT

Aim. To study *in vitro* and *in vivo* the functional suitability of ^{99m}Tc-labeled lyophilized formulation containing designed ankyrin repeat protein (DARPin) G3-$(GGGS)_3$Cys for radionuclide imaging of HER2/neu overexpression in malignant tumors.

Materials and methods. To create a targeted protein, a modified genetic construct with the sequence encoding DARPin G3-$(GGGS)_3$Cys was used. To generate the experimental probe, we used a lyophilized formulation containing DARPin G3-$(GGGS)_3$Cys with auxiliary substances and ^{99m}Tc sodium pertechnetate (500 MBq) incubated at 60 °C for 30 min. Radiochemical purity of ^{99m}Tc-G3-$(GGGS)_3$Cys was analyzed by thin-layer radiochromatography. SKOV-3, BT-474, and DU-145 cell lines were used to test binding specificity *in vitro*. The dissociation constant was determined via a saturation binding assay on SKOV-3 cells with a range of protein concentrations from 0.2 to 40 nM. Nu/j mice bearing HER2-positive SKOV-3 xenografts and HER2-negative Ramos xenografts were used to evaluate the targeting properties and biodistribution.

Results. A radiocomplex based on ^{99m}Tc and a lyophilized formulation with DARPin G3-$(GGGS)_3$Cys was obtained with the radiochemical purity of more than 96%. Binding of ^{99m}Tc-G3-$(GGGS)_3$Cys to the cells was specific (K_D 3.9 ± 0.5 nM) and proportional to the level of HER2/neu expression in the cells. The uptake of ^{99m}Tc-G3-$(GGGS)_3$Cys in SKOV-3 xenografts was significantly higher than in Ramos xenografts. ^{99m}Tc-G3-$(GGGS)_3$Cys demonstrated rapid blood and renal clearance and had low activity in the salivary glands and stomach. Liver uptake was about 5–7%ID/g. In addition, ^{99m}Tc-G3-$(GGGS_{)3}$Cys exhibited very low uptakes in the lungs, muscles, small intestine, and bones.

Conclusion. The ^{99m}Tc-labeled lyophilized formulation with DARPin G3-$(GGGS)_3$Cys is functionally suitable for imaging HER2/neu overexpression in tumors, as it binds specifically to the receptor, is stable *in vivo*, and has favorable biodistribution in organs and tissues. The radiocomplex based on ^{99m}Tc-G3-$(GGGS)_3$Cys was obtained by a simple method with high radiochemical purity.

Keywords: malignant tumors, Her2/neu, radionuclide diagnosis, DARPin G3, oxotechnetium, lyophilized formulation

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✉ *Varvashenya Ruslan N.,* mr.varvashenya@mail.ru

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Оценка функциональной пригодности лиофилизата таргетных каркасных белков с анкириновыми повторами для радионуклидной визуализации гиперэкспрессии HER2/neu в злокачественных опухолях

Варвашеня Р.Н.[1, 2], Прач А.А.[2], Плотников Е.В.[2], Деев С.М.[2, 3], Белоусов М.В.[1, 2], Ларькина М.С.[1, 2], Чернов В.И.[2, 4]

[1] Научно-образовательная лаборатория химико-фармацевтических исследований (НОЛХФИ), Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

[2] Национальный исследовательский Томский политехнический университет (НИ ТПУ)
Россия, 634050, г. Томск, 634050, пр. Ленина, 30

[3] Институт биоорганической химии (ИБХ) им. акад. М.М. Шемякина и Ю.А. Овчинникова Российской академии наук
Россия, 117997, г. Москва, ГСП-7, ул. Миклухо-Маклая, 16/10

[4] Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

РЕЗЮМЕ

Цель – изучить *in vitro* и *in vivo* функциональную пригодность лиофилизата таргетных каркасных белков с анкириновыми повторами DARPin G3-$(GGGS)_3$Cys, меченых ^{99m}Tc, для радионуклидной визуализации гиперэкспрессии HER2/neu в злокачественных опухолях.

Материалы и методы. Для наработки таргетного белка использовали модифицированную генетическую конструкцию с последовательностью, кодирующей белок DARPin G3-$(GGGS)_3$Cys. Для получения экспериментального препарата использовали лиофилизат, содержащий DARPin G3-$(GGGS)_3$Cys со вспомогательными веществами, и раствор натрия пертехнетата, ^{99m}Tc (500 МБк) при инкубации 60 °C, 30 мин. Анализ радиохимической чистоты (РХЧ) ^{99m}Tc-G3-$(GGGS)_3$Cys проводили тонкослойной радиохроматографией. Для оценки специфичности *in vitro* использовали клеточные линии: SKOV-3 > BT-474 > DU-145. Константу диссоциации определяли с помощью анализа насыщения на SKOV-3 в диапазоне концентраций белка от 0,2 до 40 нМ. Для оценки таргетных свойств и биораспределения использовали мышей линии Nu/j, несущих ксенотрансплантаты SKOV-3 (HER2/neu позитивные) и ксенотрансплантаты Ramos (HER2/neu негативные).

Результаты. Получен радиокомплекс на основе ^{99m}Tc и лиофилизата таргетных белков DARPin G3-$(GGGS)_3$Cys с РХЧ более 96%. Связывание ^{99m}Tc-G3-$(GGGS)_3$Cys с клетками является специфичным с K_D 3,9 ± 0,5 нМ и пропорционально уровню экспрессии HER2/neu в клетках. Поглощение ^{99m}Tc-G3-$(GGGS)_3$Cys в ксенотрансплантатах SKOV-3 было значимо выше, чем в ксенотрансплантатах Ramos. ^{99m}Tc-G3-$(GGGS)_3$Cys продемонстрировал быстрое выведение из крови, почечный клиренс, низкие уровни актив-

ности в слюнных железах и желудке. Уровень накопления активности в печени составил около 5–7 %ВД/г. Кроме того, ^{99m}Tc-G3-$(GGGS)_3$Cys имел очень низкое поглощение в легких, мышцах, тонком кишечнике и костях.

Заключение. Лиофилизат таргетных каркасных белков DARPin G3-$(GGGS)_3$Cys, меченый ^{99m}Tc, функционально пригоден для визуализации гиперэкспрессии HER2/neu в опухолях, поскольку специфически связывается с рецептором, стабилен *in vivo* и имеет благоприятное биораспределение в органах и тканях. Радиокомплекс 99mTc-G3-(GGGS)3Cys получен по простой процедуре с высокой радиохимической чистотой.

Ключевые слова: злокачественные опухоли, Her2/neu, радионуклидная диагностика, DARPin G3, оксотехнеций, лиофилизат

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Overexpression of transmembrane tyrosine kinase receptors, which are normally expressed on the surface of all epithelial cells in the body, often correlates with progression of malignant tumors. Human epidermal growth factor receptor type 2 (HER2/neu), which plays an essential role as an oncoprotein in malignant tumors [1] of the breast, gastrointestinal tract, ovaries, etc., is of particular interest in this process. This receptor is overexpressed in ovarian, breast, esophageal, gastrointestinal, lung, and other cancers [2].

Breast cancer (BC) is characterized by a severe disease course, low overall and recurrence-free survival, and *HER2/neu* gene amplification in 15–20% of cases. Therefore, this tumor marker is used as a target in the diagnosis and targeted therapy in patients with HER2/neu overexpression [3].

Monoclonal antibodies, antibody – drug conjugates, and tyrosine kinase inhibitors are used as targeted therapeutic agents that depend on specific recognition of HER2/neu [4]. The first step is to determine the presence and / or absence of HER2/neu overexpression on the tumor cell surface. Only after this, therapy is initiated. As a drug, trastuzumab (herceptin) is used, which is the gold standard for the treatment of patients with HER-2/neu-positive BC, significantly increasing overall and recurrence-free survival [5]. In addition, HER2/neu-targeted drugs undergo clinical evaluation for the treatment of ovarian cancer [6], non-small cell lung cancer [7], and endometrial cancer [8].

For routine use of registered drugs and further development of such therapies, it is essential to accurately determine HER2/neu expression in tumors. Expression of the targeted protein is directly related to the antitumor effect; with low expression, patients may be at risk of severe side effects following the use of targeted drugs and cytotoxins [9]. The main problem with the use of HER2/neu-targeted drugs is variability of receptor expression in malignant tumors [10].

Biopsy (immunohistochemistry and fluorescence in situ hybridization) is a routine procedure for determining HER2/neu expression [11]. However, biopsy is challenging in the context of multiple metastases due to the invasive nature of the procedure. It is virtually impossible to assess the extent of tumor progression and to detect changes in HER2/neu expression levels after neoadjuvant therapy [11]. To address the limitations of conventional biopsy, radionuclide molecular imaging of HER2/neu expression *in vivo* has been proposed as a potential solution [12].

Following the results of preclinical [13] and some clinical [12–14] trials on various types of contrast agents for molecular imaging of HER2/neu (antibodies, scaffold proteins, antibody fragments, aptamers, peptides), it can be concluded that the most promising HER2/neu-targeting molecules are scaffold proteins. They provide higher imaging contrast in a shorter time (2–4 hours after the injection) compared to other contrast agents [12].

Designed ankyrin repeat proteins (DARPins), which are engineered, high-affinity, stable proteins of small size (14–18 kDa), have the potential to be integrated into development of radiopharmaceuticals for oncoprotein imaging. DARPins with high affinity for HER2/neu were selected using ribosome display, demonstrating a clear potential for tumor targeting [15]. R. Goldstein et al. demonstrated the feasibility of radionuclide imaging of HER2/neu expression in human tumor xenografts in mice using DARPins labeled with ^{111}In and ^{125}I [16]. Further studies revealed that DARPin G3 was the optimal candidate for the development of molecular imaging agents [17].

The radionuclide most commonly employed for imaging in nuclear medicine is ^{99m}Tc (half-life 6 hours), which provides excellent spatial resolution and a low absorbed dose for patients. ^{99m}Tc is produced from ^{99}Mo generators (half-life 65.9 h), which can be delivered to remote hospitals and supply ^{99m}Tc for up to two weeks [18]. Consequently, ^{99m}Tc is an appealing candidate for SPECT imaging.

DARPin G3 labeled with [^{99m}Tc]Tc(CO)3 via a histidine-containing tag ($(HE)_3$ - tri(histidyl-glutamate)) was studied in phase I clinical trials [13]. Clinical data have demonstrated that the use of DARPin $(HE)_3$-G3 for radionuclide diagnosis is safe when patients are exposed to low doses. The diagnostic imaging of HER2-positive BC using ^{99m}Tc-labeled DARPin $(HE)_3$-G3 provided clear imaging results four hours after injection. This imaging method reliably distinguished between HER2-positive and HER2-negative tumors.

Disadvantages of this experimental radiopharmaceutical for clinical use include a complex and time-consuming two-step labeling procedure and complex purification from radiochemical impurities requiring specialized equipment. This prompted further studies to optimize and improve radioactive labeling of DARPin G3 with ^{99m}Tc for a simpler and faster one-step procedure. Previous studies utilizing DARPin G3 variants demonstrated that the use of cysteine-containing peptide-based chelators at the C-terminus to form an oxotechnetium complex resulted in low uptake in normal tissues and high uptake by tumors [19]. New DARPin G3 variants labeled with the oxotechnetium complex also provided image contrast comparable to that of clinically validated DARPin $(HE)_3$-G3 [19]. One improved variant of DARPin G3, designated ^{99m}Tc-G3-$(GGGS)_3$Cys, contains a Gly-Gly-Gly-Ser-Cys chelator conjugated via the - $(Gly-Gly-Gly-Ser)_2$ linker at the C-terminus. This variant has been proposed for pilot clinical trials (NCT05923268).

Prior to commencing clinical trials, it is essential to ascertain the functional suitability of DARPin G3-$(GGGS)_3$Cys with its complete sequence in the form of a lyophilized formulation. A number of factors influence the functional suitability of a targeted protein molecule, including the choice of a chelating agent for ^{99m}Tc binding, labeling conditions, and composition (combination of chemical precursors). The type of a dosage form plays an essential role in affinity, targeting properties, and *in vivo* biodistribution of such molecules. Any alterations to the composition of the dosage form, including transition from a solution to a lyophilized formulation, may result in a reduction or complete loss of its functional suitability for HER2/neu imaging. Consequently, it is necessary to conduct *in vitro* and *in vivo* experiments to evaluate these properties.

It is worth noting that cysteine-containing proteins are susceptible to oxidation to non-affinity homodimers, which makes them highly sensitive to technological processes employed in the production of lyophilized formulations. These are undoubtedly the most convenient and storage-stable dosage forms for routine production of radiopharmaceuticals in a medical organization [18].

The aim of this research was to assess *in vitro* and *in vivo* the functional suitability of a ^{99m}Tc-labeled lyophilized formulation containing DARPin G3-$(GGGS)_3$Cys for radionuclide imaging of HER2/neu overexpression in malignant tumors.

MATERIALS AND METHODS

The nucleotide sequence of the *DARPin G3* gene was deduced from the amino acid sequence for DARPin G3 in the PDB (PDB access number:

2JAB) taking into account codons most common in highly expressed *Escherichia coli* genes using the freely available DNA builder software (http://www.innovationsinmedicine.org/software/DNABuilder/). The genome was assembled by the polymerase chain reaction (PCR) from chemically synthesized 50-bp long oligonucleotides with partially complementary sequences. Expression, isolation, and purification of DARPin G3-(GGGS)3Cys were performed according to the previously described method [19].

The method for producing the experimental formulation. ^{99m}Tc pertechnetate [^{99m}Tc]TcO4 was obtained from the commercial ^{99}Mo / ^{99m}Tc GT-4K generator (Karpov Institute of Physical Chemistry, Obninsk, Russia). Samples were obtained using chemically pure reagents from various suppliers, including Fluka, Acros Organics (UK), Panreac, Sigma Aldrich (USA), and others. In a lyophilized mixture containing 3.3 mg DARPin G3-$(GGGS)_3$Cys, 0.66 mg D-mannose, 0.33 mg PEG-4000, 0.075 mg tin (II) chloride dihydrate, 5 mg sodium gluconate, 0.1 mg ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, and phosphate buffer, 500 µl sodium ^{99m}Tc pertechnetate solution with the activity of 500 MBq was added and mixed. The contents of the vial were incubated at 60 °C for 30 minutes. Then the contents of the vial were diluted with a sterile 0.9% sodium chloride solution to 10 ml. Subsequently, the solution was purified using a sterile syringe filter with a pore size of 0.2 µm into a depyrogenated sterile vial. The radiochemical purity of the experimental ^{99m}Tc-G3-$(GGGS)_3$Cys samples was analyzed by thin-layer chromatography using ITLC SG strips (Aglient Technologies, Inc., Folsom, USA) in the phosphate buffered saline (pH = 7.4). Radioactivity on ITLC strips was recorded in counts per minute (CPM) using the miniGITA Single radio-TLC system (Elysia Raytest, Germany). In order to obtain a sterile solution for experiments on laboratory animals, the radioactivity was measured on the radiometer (Amplituda, Russia) equipped with an ionization chamber.

Radioactivity in both *in vitro* and *in vivo* samples was quantified using the automated Wizard 2480 Gamma Counter (Pelkin Elmer, USA). HER2/neu-expressing human cancer cell lines SKOV-3 (human ovarian carcinoma) and BT-474 (human breast carcinoma), as well as DU-145 cells (human prostate adenocarcinoma) with low HER2/neu expression were purchased from PrimeBioMed LLC (Moscow, Russia). The cells were cultured in the Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU / ml penicillin, and 100 µg / ml streptomycin in the humidified incubator with 5% CO_2 at 37 °C.

In order to assess the specificity of the *in vitro* assay, the cells were seeded into 6-well plates at a seeding density of 7×10^5 cells per well 24 hours prior to the experiment. One plate was utilized for each cell line. A 100-fold excess of unlabeled DARPin G3-$(GGGS)_3$Cys was used as a control group. The same volume of cell culture medium was added to three petri dishes of the experimental group. The petri dishes were incubated at 37 °C for 30 minutes to saturate the HER2/neu receptors. Subsequently, the [^{99m}Tc]Tc-G3-$(GGGS)_3$Cys solution was added to each petri dish to a final concentration of 1 nM, and the samples were incubated at 37 °C for 1 h. Following this incubation period, the medium was collected, the cells were washed with the phosphate buffer, and the solutions were pooled. The cells were then detached with trypsin and collected. Radioactivity in every fraction was measured by the gamma counter, and the percentage of cell-associated activity per 1 million cells was calculated. The experiment was conducted in triplicate.

The method for determining the equilibrium dissociation constant (C_D) was described previously [19]. The experiment was performed on SKOV-3 cells. Radioactivity was measured using the gamma counter. C_D parameters and the maximum number of binding sites per cell (Bmax) were calculated by nonlinear regression using the Prism software (GraphPad Software, USA).

To assess the targeting properties and biodistribution of the ^{99m}Tc-labeled lyophilized formulation containing G3-(GGGS)3Cys, immunodeficient Nu/j mice were used bearing HER2-positive SKOV-3 xenografts and HER2-negative Ramos xenografts. Subcutaneous implantation of 10 million SKOV-3 cells or the same number of Ramos cells was performed in female Nu/j mice. The experiments were conducted three weeks after the implantation. The mean weight of the animals at the time of the experiment was (25.4 ± 1.8) g. The mean tumor weight was (0.4 ± 0.2) and (0.2 ± 0.05) g for SKOV-3 and Ramos xenografts, respectively.

The mice were administered 3 μg of ^{99m}Tc-G3-(GGGS)$_3$Cys (40 kBq, 100 μl in sterile phosphate buffered saline) via the tail vein. Following euthanasia of the animals, their blood, organs, and tissues of interest were collected and weighed. The radioactivity was then measured using the gamma counter. The uptake in organs was calculated as a percentage of the injected dose per gram of the sample (%ID/g).

When planning and conducting animal experiments, we adhered to all applicable international and national guidelines for the care and use of animals for scientific purposes. The animal research protocol was approved by the Ethics Committee at Siberian State Medical University (Protocol code 7715, 20190826).

The Mann – Whitney *U*-test was used to determine significant differences ($p < 0.05$). Statistical analysis was performed using the Prism software (version 9.0.0 for Windows; GraphPad Software, USA).

RESULTS AND DISCUSSION

The composition of the lyophilized formulation with G3-(GGGS)$_3$Cys for the production of the experimental probe was multicomponent due to the necessity of using two groups of excipients [19, 21]. The first group of excipients was designed to safeguard the functionality of the protein during lyophilization and to maintain its capacity of transition into a solution upon dissolution of the lyophilized material. D-mannose and PEG-4000 were employed as such excipients. The second group of excipients was essential for the radioactive labeling of the protein. This process is based on the reduction of heptavalent ^{99m}Tc sodium pertechnetate to a pentavalent substance, followed by the formation of an oxotechnetium complex with a chelating group (-GGSC). The reducing agents employed were tin (II) chloride, sodium gluconate, and EDTA. After adding the pertechnetate eluant from the generator to the lyophilized formulation, it was then incubated in accordance with the procedure outlined in [19]. The radiochemical purity of the ^{99m}Tc-G3-(GGGS)$_3$Cys radiocomplex was 98 ± 1 %. The method for labeling the G3-(GGGS)$_3$Cys solution, previously described in [19], was replicated for the lyophilized formulation in order to obtain the ^{99m}Tc-G3-(GGGS)$_3$Cys radiocomplex of high purity, which does not require purification.

In order to study the binding specificity of the ^{99m}Tc-labeled G3-(GGGS)$_3$Cys to HER2/neu, cell lines with varying levels of receptor expression were employed: SKOV-3, BT-474, and DU-145. The experiment was conducted by blocking the receptor with unlabeled G3-(GGGS)$_3$Cys. The study of binding specificity *in vitro* demonstrated high specific binding to cells, which was proportional to the level of HER2/neu expression in the cells. Blocking the receptors with excess unlabeled protein showed a significant decrease in ^{99m}Tc-G3-(GGGS)$_3$Cys binding in all groups of cells ($p < 0.0005$) (Fig. 1).

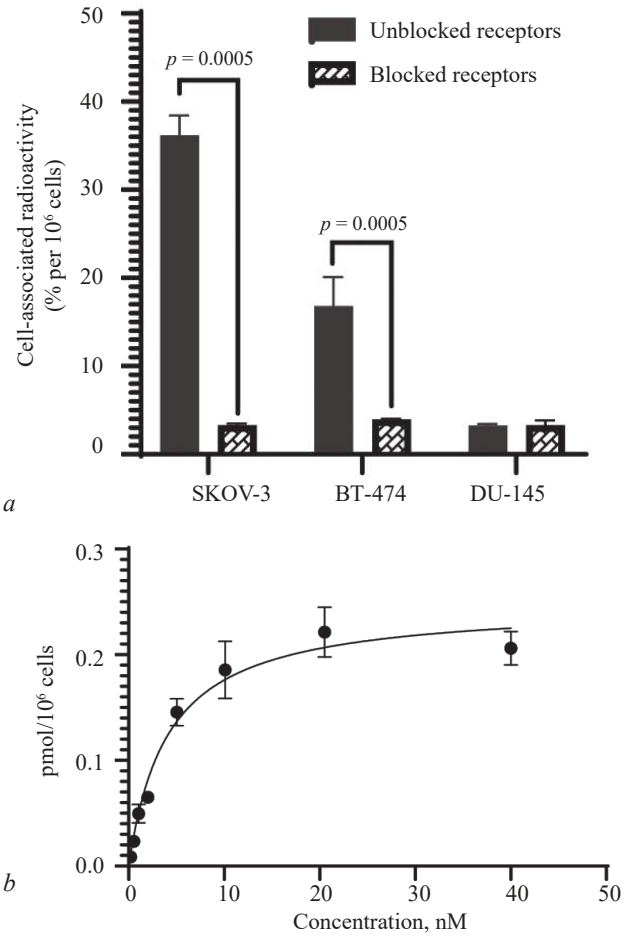

Fig. 1. Results of the *in vitro* assessment of the ^{99m}Tc-labeled lyophilized formulation with G3-(GGGS)3Cys: *a* – determination of binding specificity to HER2/neu; *b* – saturation curve on HER2/neu-expressing SKOV-3 cells. The results are presented as the mean % per 10^6 cells ± standard deviation from three samples.

Affinity assessment by the binding saturation assay demonstrated that ^{99m}Tc-G3-(GGGS)$_3$Cys binds to HER2/neu receptors on the surface of SKOV-3 cells with a nanomolar C_D (3.9 ± 0.5 nM) (Fig. 1), thereby confirming high affinity of the ^{99m}Tc-labeled protein obtained from the lyophilized formulation for its target receptors.

A comparison of the biodistribution and accumulation of ^{99m}Tc-G3-(GGGS)$_3$Cys in SKOV-3 xenografts with high HER2/neu expression and Ramos xenografts with no HER2/neu expression is presented in Figure 2. A parallel comparison of the biodistribution of ^{99m}Tc-G3-(GGGS)$_3$Cys in the SKOV-3 and Ramos xenografts revealed that the pattern of biodistribution was similar ($p > 0.05$), with the exception of radioactivity accumulation in the tumor. The accumulation of ^{99m}Tc-G3-(GGGS)$_3$Cys in the SKOV-3 xenografts was found to be significantly higher ($p < 0.005$) than in the Ramos xenografts. This indicates that the level of accumulation correlates with HER2/neu expression.

The radiopharmaceutical ^{99m}Tc-G3-(GGGS)$_3$Cys exhibited rapid blood clearance, predominantly via the kidneys. Additionally, low retention of radioactivity in the kidneys was observed (12–20% ID / g). This may be attributed to the rapid internalization of the protein following reabsorption in the kidneys and subsequent excretion of radiocatabolites containing glycine amino acid residues from the cell [17].

Low radioactivity accumulation was further observed in salivary glands and stomach, indicating the stability of the radiocomplex *in vivo*, since no hydrolysis of the complex was noted with the release of free ^{99m}Tc capable of accumulating in these organs. Furthermore, ^{99m}Tc-G3-(GGGS)$_3$Cys exhibited minimal uptake in the lungs, muscles, small intestine, and bones, which is favorable for imaging metastases in these areas [22].

Uptake in the liver was approximately 5–7% ID / g, with the tumor-to-liver ratio of less than one (approximately 0.7 times), which is suboptimal for imaging of liver metastases. Nevertheless, it should be noted that the biodistribution in mice is not entirely comparable to that observed in humans. Consequently, the uptake in the liver is unlikely to interfere with imaging of liver metastases in clinical trials [23]. It appears that biliary excretion of radiometabolites played a minor role, as radioactivity in the gastrointestinal tract with its contents was low (approximately 2%).

It can be supposed that the mechanism of radioactivity retention in the liver is not solely due to hepatobiliary excretion, but may also be influenced by ligand – receptor interactions or other mechanisms. HER2/neu expression takes place in the liver, which may contribute to the accumulation of G3-(GGGS)$_3$Cys radioactivity in this organ. The selection of the optimal protein dose in clinical trials of radiolabeled scaffold proteins allows to control this process and attain the desired contrast for visualization of liver metastases [14, 24].

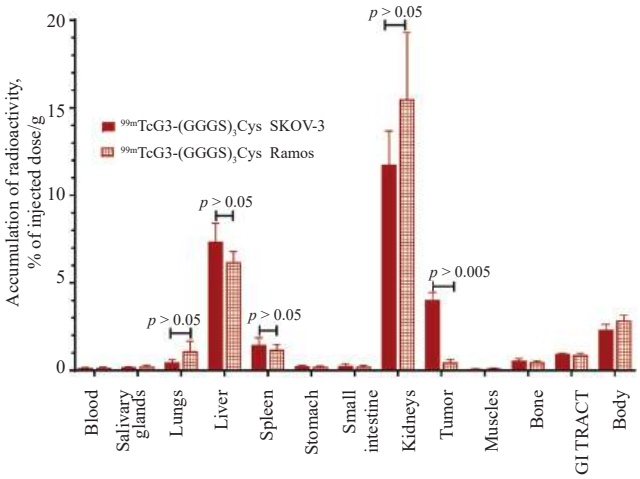

Fig.2. Comparative biodistribution of ^{99m}Tc-G3-(GGGS)$_3$Cys 4 hours after injection in HER2/neu-positive SKOV-3 and HER2/neu-negative Ramos xenografts in Nu/j mice. The data for five mice are presented as the mean % ID / g ± standard deviation. The data for the gastrointestinal tract with its contents and the rest of the body are presented as % ID for the whole sample

Since there was no direct comparison in the same batch of mice of ^{99m}Tc-G3-(GGGS)$_3$Cys obtained from the lyophilized formulation and ^{99m}Tc-G3-(GGGS)$_3$Cys obtained from the protein solution without technological interventions, it is not possible to state with any degree of reliability whether there are any changes in the functional suitability of the protein obtained from the lyophilized formulation. However, a comparison with the previously published data [19] on biodistribution and targeting properties in Nu/J mice bearing SKOV-3 xenografts revealed that the protein obtained from the lyophilized formulation in the selected composition exhibits properties identical to the native protein.

CONCLUSION

The studies *in vitro* and *in vivo* confirmed the functional suitability of the ^{99m}Tc-labeled lyophilized formulation with DARPin G3-$(GGGS)_3$Cys for radionuclide imaging of HER2/neu overexpression in malignant tumors. The radiocomplex based on ^{99m}Tc-G3-$(GGGS)_3$Cys is obtained by a simple method with high radiochemical purity, binds specifically to HER2/neu in tumor cells, is stable *in vivo*, and has favorable biodistribution in organs and tissues for subsequent transfer into clinical trials.

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Authors' contribution

Varvashenya R.N. – carrying out of radiochemical studies in vivo, in vitro, drafting of the article. Prach A.A. – carrying out of the experiments in vivo, in vitro. Plotnikov E.V., Larkina M.S. – conception and design, interpretation of the data, critical revision of the manuscript for important intellectual content, drafting of the article. Deev S.M. – conception and design. Belousov M.V. – interpretation of the data, critical revision of the manuscript for important intellectual content. Chernov V.I. – justification of the manuscript, conception and design, interpretation of the data, critical revision of the manuscript for important intellectual content, drafting of the article.

Authors' information

Varvashenya Ruslan N. – Post-Graduate Student, Laboratory Researcher, Science and Education Laboratory for Chemical and Pharmaceutical Research, Siberian State Medical University, Tomsk, mr.varvashenya@mail.ru, http://orcid.org/0009-0002-4282-3198

Prach Anastasia A. – Post-Graduate Student, Research School of Chemical and Biomedical Technologies, National Research Tomsk Polytechnic University, Tomsk, nastya.prach@mail.ru, http://orcid.org/0000-0002-6975-2361

Plotnikov Evgeny V. – Cand. Sci. (Chemistry), Associate Professor, Research School of Chemical and Biomedical Technologies, Tomsk Polytechnic University, Tomsk, plotnikovev@tpu.ru, ORCID: http://orcid.org/0000-0002-4374-6422

Deev Sergey M. – Dr. Sci. (Biology), Professor, Academician of the Russian Academy of Sciences, Head of the Laboratory for Molecular Immunology, Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry, Moscow, biomem@mail.ru, http://orcid.org/0000-0002-3952-0631

Belousov Mikhail V. – Dr. Sci. (Pharmacy), Professor, Head of the Pharmaceutical Analysis Division, Siberian State Medical University, Tomsk, belousov.mv@ssmu.ru, http://orcid.org/0000-0002-2153-7945

Larkina Maria S. – Dr. Sci. (Pharmacy), Professor, Pharmaceutical Analysis Division, Siberian State Medical University, Tomsk, marialarkina@mail.ru, ORCID: http://orcid.org/0000-0003-1176-2441

Chernov Vladimir I. – Corresponding Member of the Russian Academy of Sciences, Dr. Sci. (Med.), Professor, Head of the Department of Radionuclide Diagnosis, Cancer Research Institute of Tomsk NRMC, Tomsk, chernov@tnimc.ru, http://orcid.org/0000-0001-8753-7916

(✉) **Varvashenya Ruslan N.,** mr.varvashenya@mail.ru

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