In Vitro Evaluation of a Specific Radiochemical Compound Based on 99mTc-labeled DARPinG3 for Radionuclide Imaging of Tumors Overexpressing Her-2/neu

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Abstract. It is still necessary to search for new informative diagnostic methods to detect malignant tumors with overexpression of Her-2/neu, which are characterized by the aggressive course of the disease, rapid rate of tumor growth and low rates of relapse-free and overall survival. In recent years, the radioisotope techniques for detection of specific tumor targets have been developing actively. *Purpose*: to develop a chemically stable radiochemical compound for the targeted imaging of cells overexpressing Her-2/neu. *Material and methods*: The study was performed using 2 cell lines. The human breast adenocarcinoma HER2-overexpressing cell line BT-474 was chosen to detect specific binding. As a control, HER2-negative human breast adenocarcinoma MCF-7 was used. The human breast adenocarcinoma BT-474 and MCF-7 cell lines were seeded in chamber-slides at the density of 35,000 cells/ml in trypsin–EDTA (PanEco) medium and grown overnight at 37°C. After that both cell lines were washed with Phosphate buffered saline (PBS) and distributed into test tubes to 1 ml (5 millions cells in each). After adding 100 µl (70 MBq) studied complex of 99mTc–DPAH–DARPinG3 was incubated for 40 min at +4°C. Washing was performed three times with buffer PBS and 5% Bovine Serum Albumin (BSA). The characteristics of the binding specificity of the test set with the HER-2/neu receptor were determined by direct radiometric and planar scintigraphy. Nonparametric Mann-Whitney test was used to assess the differences in the quantitative characteristics between groups. *Results*: The output of the labeled complex was more than 91%, with a radiochemical purity of more than 94%. When carrying out a visual scintigraphic assessment much greater intensity accumulation of radiotracer was observed in the studied cell culture surface receptor overexpressing Her-2/neu. The results of direct radiometric also showed higher accumulation of the radiopharmaceutical in the adenocarcinoma cell line BT-474 human breast cancer overexpressing Her-2/neu compared to the control group. *Conclusion*: The preclinical studies demonstrated a high in vitro stability of the study compound, as well as its accumulation in the cell group overexpressing Her-2/neu.

Recently, a personified approach has been widely used in the cancer treatment, which consists in the selection of a particular category of patients, who would most likely have a response to the treatment according to their "molecular profile". Target therapy is the most demonstrative example of this approach in the treatment of oncological patients. Target therapy affects molecular targets which are key to the vital activity of tumor cells with a minimal effect on normal tissue cells [1, 2].

Over the past two decades a particular interest has been manifested to the study of surface receptor Her-2/neu, a member of transmembrane tyrosine kinase receptors. On the one hand, overexpression of Her-2/neu is a marker of unfavourable prognosis of the disease, which is manifested by the aggressive course of the disease, rapid rate of

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tumor growth and low rates of relapse-free and overall survival. On the other hand, overexpression of Her-2/neu is an indication for the target therapy with Herceptin which is still the standard of treatment for patients with Her-2/neu-positive breast cancer [3, 4].

Currently, there are a number of diagnostic techniques for determination of the Her-2/neu status. The immunohistochemical method is often used, although it has a number of drawbacks: the necessity of an invasive procedure (taking a biopsy or surgical material), a high incidence of false positive and false negative results, as well as a possible violation in methodology and misinterpretation of results [5, 6].

At present, molecular genetics diagnostic methods are being introduced and include fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH). However, they also have several limitations which consist of the inability to estimate the tumor process in vivo and inaccessibility to perform the research in most histopathology laboratories. Some of uncertain cases are associated with heterogeneity of the HER2 expression that is a serious diagnostic problem for the accurate determination of the receptor status of the tumor.

In this regard, it is still necessary to search for new informative diagnostic methods for detection of malignant tumors with overexpression of Her-2/neu. In recent years, the radioisotope techniques are developing for detection of specific tumor targets [7]. One of the most studied components for the formation of specific relations with the isotopes are "targeting" non-immunoglobulin molecules DARPins (designed ankyrin repeat proteins), which belong to a novel class of binding molecules with the potential to overcome the limitations of monoclonal antibodies, hence allowing novel therapeutic approaches. The main advantages of such protein structures are a small size (14–20 kDa), a stable structure, high specificity to bind with a target, high stability of conjunction, low immunogenicity as well as a much lower cost of production [8, 9].

Technetium-99m (99mTc) is the most widely used radionuclide for radiopharmaceuticals, which is characterized by availability, short half-life (T1/2 = 6.05 h), low cost and operation of 99mTc generators, as well as simple technology of obtaining eluate from the generator.

The chelating agent is an important component in a radiochemical synthesis for binding with 99mTc, as well as for the development of a technique for chemical modification of recombinant address molecules with the retaining their ability to bind with specific receptors of tumor cells [10–12].

MATERIAL AND METHODS

DARPinG3 was amplified from plasmid pCG-Hnse-DARPin-d18-G3 using specific primers 5'-cgccgaattctt-gcaggtttcagccag by our colleagues from the M.M. Shemyakin–Yu.A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences.

Technetium eluate for research was obtained from technetium generators "99mTc-GT-TOM". Pertechnetate ions from the initial generator eluate have a higher degree of oxidation (VII) and high chemical activity without tendency to complex formation. Various reducing agents are used for reducing their oxidation state. In our work, stannous chloride ($SnCl_2 \cdot 2H_2O$) was used as the reducing agent.

In this study, we used succinimid-1-yl 6-(bis(pyridin-2-ylmethyl)amino)hexanoate (DPAH-NHS ester) as the chelating agent.

PREPARATION OF RADIOPHARMACEUTICAL

To mixture a radiopharmaceutical 100 µl of 0.5 mg/ml of DPAH–DARPinG3 solution (in phosphate buffer), 60 µl sodium citrate solution at a concentration of 100 mg/ml, 20–60 µl of a solution of $SnCl_2$ $2H_2O$ at a concentration of 7 mg/ml and 1 ml of a 99mTc eluate with activity of 0.5–3.7 GBq were added in a 10 cc (cubic centimeter) bottle without inserting an air needle. The radiopharmaceutical is ready for use after mixing and incubation for 30 min at room temperature.

The radiochemical purity (RCP) and radiochemical yield (RCY) of the produced pharmaceuticals were determined by the method of thin layer chromatography (TLC), using plates PTLC silicagel-AF-A-UV (Sorbfil). Two systems were used as mobile phases: 1) acetone and 2) $C_2H_5OH : 25\%\ NH_4OH : H_2O \div 2 : 5 : 5$. With acetone, the free pertechnetate migrates with the solvent front, whereas colloidal 99mTc and 99mTc–DPAH–DARPinG3 both remain at the application point. With mix (2) as the mobile phase, colloidal 99mTc remains at the application point, whereas free pertechnetate and 99mTc–DPAH–DARPinG3 move with the solvent front. The activity distribution of 99mTc on radiochromatograms was studied using the GammaScan-01A setup.

CELL LINES AND INCUBATION CONDITION

The human breast adenocarcinoma HER2-overexpressing cell line BT-474 (Russian Cell Culture Collection) was chosen to detect specific binding. As a control, HER2-negative human breast adenocarcinoma MCF-7 (Russian Cell Culture Collection) was used.

CELL LINES RADIOLABELING

The human breast adenocarcinoma BT-474 and MCF-7 cell lines were seeded in chamber-slides at the density of 35,000 cells/ml in trypsin–EDTA (PanEco) medium and grown overnight at 37°C. After that both cell lines were washed with phosphate buffered saline (PBS) and distributed into test tubes to 1 mL (5 millions cells in each). After adding 100 µl (70 MBq) studied complexes of 99mTc–DPAH–DARPinG3 and 99mTc–DARPinG3, tubes were incubated for 40 min at +4°C. Washing was performed three times with buffer PBS and 5% Bovine Serum Albumin (BSA).

Activity measurement was performed with a radiometer for in vitro studies ("Amplituda" 2008 YG). Visual scintigraphic and quantitative evaluation was performed using a SPECT ("E.CAM180"), processing the data was carried using specialized software package (Esoft 5.5 Siemens, Germany).

Statistical evaluation of the results was performed using the software package of "STATISTICA" for Windows.

RESULTS AND DISCUSSION

The results of binding DPAH–DARPinG3 with technetium-99m depending on the amount of relucting agent are showed in Table 1. As can be seen from the presented data, the largest radiochemical yield with a low impurity content of unreduced and hydrolyzed technetium-99m was observed in case of adding 50 µl solution of stannum chloride to 100 µl solution of DPAH–DARPinG3 and 60 µl sodium citrate solution, it was more than 91.0%.

The radiochemical yield was dropping as a large amount of stannum chloride was added due to the increase of formed hydrolyzed technetium-99m colloid. Thus further in vitro studies were performed with a compound having the following composition: 100 µl solution of DPAH–DARPinG3 in a concentration of 0.5 mg/ml (in phosphate buffer), 60 µl solution of sodium citrate in a concentration of 100 mg/ml, 50 µl solution of $SnCl_2 \cdot 2H_2O$ with a concentration of 7 mg/ml and 99mTc with activity of 0.5–3.7 GBq.

The main mechanism of action of the specific compound based on recombinant target molecules DARPinG3 consists in their interaction with the receptors on the surface of tumor cells, which makes it possible to detect the malignancies with overexpression of the Her-2/neu with high sensitivity and specificity.

The human adenocarcinoma cell line BT-474 with overexpression of Her-2/neu was selected to evaluate the specificity of the radiopharmaceutical`s accumulation in the study. The evidence of selective interaction with the radiopharmaceutical is a high accumulation on the surface of the group of cells overexpressing Her-2/neu.

Much higher intensity of the accumulation of the studied radiopharmaceutical was detected in the cells' culture with overexpression of the Her-2/neu during a visual scintigraphic evaluation.

In direct radiometry, a higher accumulation of the radiopharmaceutical was revealed also in the human BT-474 mammary adenocarcinoma cell line with overexpression of the Her-2/neu (Table 1).

TABLE 1. Accumulation of radiopharmaceutical "99mTc–DPAH–DARPinG3" в cell lines BT-474 и MCF-7

No. experiment	Cell bindings (M ± SD)		*p*
	MCF-7, Mbc	BT-474, Mbc	
1	0.098 + 0.014	0.306 + 0.038	0.043
2	0.072 + 0.021	0.41 + 0.025	0.038
3	0.087 + 0.019	0.29 + 0.042	0.019
4	0.069 + 0.022	0.31 + 0.039	0.025
5	0.091 + 0.015	0.402 + 0.051	0.003

M—mean value, SD—standard deviation, *p*—the level of significance of the differences.

CONCLUSION

A new specific compound based on recombinant target molecules “99mTc–DPAH–DARPinG3” was studied, a procedure for its preparation was described, and its specificity and affinity for the Her’s-2/neu receptor were characterized. High specificity and stability of this compound make it a promising agent for the diagnosis of oncological diseases with overexpression of the Her-2/neu.

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