A Targeted Toxin Based on a Binding Module of Non-Immunoglobulin Scaffold as a New Agent for Theranostics

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The aim of the investigation was to create multifunctional antitumor protein agent DARPin-mCherry-PE40 possessing both diagnostic and therapeutic properties.

Materials and Methods. The experiments were carried out on human cell lines of breast adenocarcinoma SK-BR-3. Chinese hamster ovarian cell line CHO-K1 was used as a control. Genetic engineering methods, metal chelate affinity chromatography, confocal microscopy and spectrophotometric MTT test were used to perform the experiments.

Results. A multifunctional targeted anticancer protein agent DARPin-mCherry-PE40 consisting of targeted HER2-specific non-immunoglobulin module DARPin, an imaging module mCherry and cytotoxic module based on a fragment of Pseudomonas exotoxin A (PE40) has been created. Using HER2-positive cells it was shown *in vitro* that all three domains of the recombinant protein retained their functional qualities — high affinity for HER2 antigen, ability to fluorescence, and cytotoxicity.

Conclusion. The ability of the targeted antitumor agent DARPin-mCherry-PE40 selectively stains HER2-positive cells and highly selective cytotoxicity against these cells make this targeted recombinant protein DARPin-mCherry-PE40 a perspective theranostic agent for the diagnosis and treatment of HER2-positive human tumors.

Key words: DARPin; HER2 receptor; mCherry; *Pseudomonas aeruginosa* exotoxin A fragment; PE40; theranostics.

At present the development of new methods and approaches for highly sensitive detection of tumors, as well as creation of new compounds for their highly selective therapy is the most burning and actively developing direction in biology and medicine. The science combining these two directions — theranostics — is a new strategy in medicine, the main idea of which lies in creation of preparations enabling physicians to simultaneously visualize the disease, exert a therapeutic effect on it, observe the kinetics of drug delivery to the tumor, and adjust the scheme of therapy in the process of monitoring [1, 2].

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Searching for molecular targets for diagnosis and treatment of oncological diseases, determining molecular profile of a cancer cell is another important direction of investigations being a constituent part of theranostics.

One of the most examined tumor molecular targets is human epidermal growth factor receptor 2 (HER2). Tyrosine kinase receptor HER2 is normally present on all epithelial human tissues and its density amounts to several thousand molecules per cell. When the status of a cell is normal, HER2 is known to form functionally active heterodimers with other receptors of transmembrane tyrosine kinase family (HER3 and HER4), being in a complex with the ligand [3]. In case of malignant cell transformation amplification of *HER2* gene takes place, leading to overexpression of the receptor encoded by it. Concentration of HER2 on the cell surface in such situation sharply increases — up to several million molecules per cell, and HER2 acquires the ability to constitutive heterodimerization with HER3 (even if HER3 does not interact with its ligand) [4]. Continuous signal transduction from the membrane to the cell nucleus results in the increase of cell proliferation, inhibition of apoptosis and, eventually, to tumor formation and metastasis. It is known, that 15–20% of breast tumors and human ovarian carcinoma are characterized by the elevated level of *HER2* gene expression [5–7]. In modern medical practice HER2 oncomarker is a therapeutic target for monoclonal antibodies (pertuzumab, trastuzumab) and kinase inhibitors in the treatment of HER2-positive breast tumors [8]. In order to enhance a cytotoxic effect, antibodies or their fragments are conjugated with toxins, for example diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A (PE40), obtaining immunotoxins [9, 10].

However, antibodies used for cytotoxic module delivery are characterized, as a rule, by a low level of expression, tendency to aggregation and a limited penetration into the tissues. In this connection an active search for new targeted molecular constructions — scaffold proteins, which are alternative to binding antibody domains, is going on.

Some years ago in the laboratory of prof. Plünckthun (Zurich, Switzerland) a novel class of targeting molecules of non-immunoglobulin nature based on artificial proteins with ankyrin repeats — DARPins (designed ankyrin repeat proteins) — was developed [11]. DARPins do not contain cysteine residues in their scaffold enabling production of these proteins directly in the cytoplasm of *Escherichia coli*, possess a high level of expression in the bacterial system, are monomers in the solution, resist against aggregation, and are highly stable to proteases [12–14]. All these features give the scaffold proteins great advantages over immunoglobulins as alternative targeted components in the structure of multifunctional compounds designed for diagnosing and therapy of various diseases.

Considering the merits of targeted modules based on DARPin relative to antibodies, we used DARPin_9-29 specific to subdomain I of HER2 extracellular domain (K_D=3.8 nM) in our work [15]. We used DARPin_9-29 as a targeted module for delivery a cytotoxic module (based on truncated variant of *Ps. aeruginosa*; exotoxin A lacking the N-terminal cell binding domain (252–613 a.a., 40 kDa)) and an imaging module (based on fluorescent protein mCherry) to HER2-positive cancer cells [16].

The aim of the investigation was to create a multifunctional antitumor protein agent DARPin-mCherry-PE40 possessing both diagnostic and therapeutic functions.

Materials and Methods

Obtaining a genetic construction pDARP-mCherry-PE40. pDARP-PE40 plasmid, containing encoding sequences of the targeted module DARPin_9-29 and a cytotoxic module based on PE40 fragment of Pseudomonas exotoxin A in one reading frame under the control of inducible promoter T7 has been previously created by us [17]. Encoding sequence of the fluorescent protein mCherry was obtained by treating pIG6-4D5scFv-mCherry with AscI restriction endonuclease [18] and ligated with plasmid pDARP-PE40 [17], pretreated with the same endonuclease. Plasmid pDARP-mCherry-PE40 containing in one reading frame under the control of inducible promoter T7 encoding sequences of the targeted module DARPin_9-29, fluorescent protein mCherry, and PE40 fragment of Pseudomonas exotoxin A (Figure 1 (a)) was obtained. Selection of transformants carried the plasmid was performed by means of restriction endonuclease mapping. After sequence verification the plasmid pDARP-mCherry-PE40 was used for protein expression.

Expression and purification of the recombinant protein pDARPin-mCherry-PE40. E.coli BL21(DE3) strain was transformed by pDARP-mCherry-PE40 plasmid. Fresh transformants (1 colony per 1 ml of medium) was inoculated in 25 ml of medium, containing 2 mM $MgSO_4$, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 45 mM K_2HPO_4, 100 mM NaCl, 1% yeast extract, 1% tryptone, 0.1 g/L ampicillin, and cultivated in 250 ml flask at 37°C till the culture achieved the optical density of OD_{600} 0.5. Then the incubation temperature was lowered to 13°C, isopropyl thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was grown under vigorous aeration till the optical density of OD_{600} 18–20 was reached. The cells were harvested by centrifuging on a cooled centrifuge (4°C) at 6,000 g for 10 min. The cell pellet was resuspended in 10 ml of buffer for lysis (20 mM Na phosphate; 100 mM NaCl; pH 7.5; 60 μg/ml lysozyme). Then the cells were sonicated on ice using Vibra Cell disintegrator (Sonics, USA) in the following mode: 10 s of ultrasound processing, 10 s of cooling, 30 cycles in all. Cellular debris was removed by centrifugation at 15,000 g for 20 min on a cooled centrifuge (4°C). PMSF protease inhibitor (1 mM) was added to the clarified supernatant. The lysate was passed through 0.22 μM filter, imidazole (up to final concentration of 30 mM) and NaCl (up to 500 mM final concentration) were added, and applied on Ni^{2+}-NTA column (GE Healthcare, USA) equilibrated by buffer: 20 mM Na phosphate; pH 7.5; 500 mM NaCl;

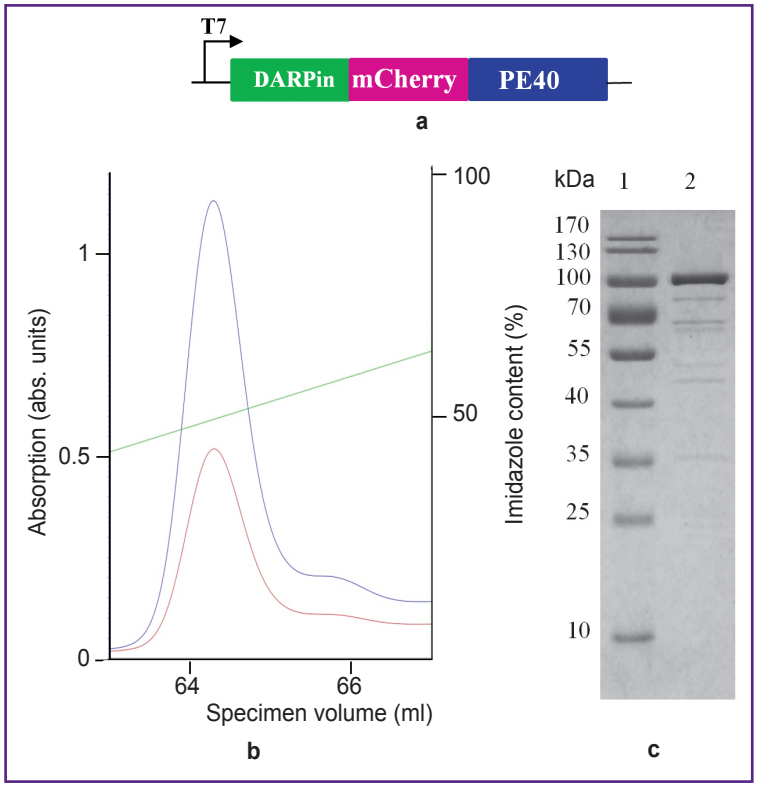

Figure 1. Expression and purification of targeted hybrid toxin DARPin-mCherry-PE40: (a) a scheme of genetic construction for bacterial expression of recombinant protein DARPin-mCherry-PE40 (T7 promoter, encoding sequences of targeted module PARPin, fluorescent mCherry module and cytotoxic PE40 module are designated on the scheme); (b) purification of DARPin-mCherry-PE40 protein with the help of metal chelate affinity chromatography (profile of the targeted protein elution by a linear imidazole gradient, 30–500 mM); (c) gel electrophoresis of DARPin-mCherry-PE40 in 12% polyacrylamide gel. Line *1:* molecular weight marker (PageRuler Prestained Protein, Thermo Fisher Scientific, USA), line *2*: purified fraction of DARPin-mCherry-PE40 protein

30 mM imidazole. Targeted protein DARPin-mCherry-PE40 was eluted using imidazole linear gradient (30–500 mM) (Figure 1 (b)). The peak registered at ~150 mM of imidazole was used for the cell work. The yield of the purified protein was 35 mg per 1 L of culture.

Cell lines and cultivation conditions. Cultivated human breast adenocarcinoma cell line SK-BR-3 (ATCC, cat. #HTB-30) overexpressing HER2, and Chinese hamster ovarian cell line CHO-K1 (ATCC, cat. #CCL-61) were grown in McCoy's 5A medium (Life Technologies, USA) with 10% fetal bovine serum (Hyclone Europe, Belgium) in the atmosphere of 5% CO_2 at 37°C. To prevent enzymatic removal of surface receptors during subcultivation, cells were detached from the substrate by Versene solution (PanEco, Russia) without trypsin application.

Investigation of cytotoxicity of DARPin-mCherry-PE40. To determine the cytotoxicity of the targeted toxin DARPin-mCherry-PE40, cells were seeded on 96-well plates (Corning, USA) at the density of $4 \cdot 10^3$ (SK-BR-3) or $1.5 \cdot 10^3$ (CHO-K1) cells per well and cultivated overnight. Then the growing medium in the wells was replaced by a fresh one containing different concentrations of the recombinant targeted toxin DARPin-mCherry-PE40 and incubated at 37°C in the atmosphere of 5% CO_2 for 72 h.

Cell viability was assessed by MTT method [19]. Relative cell viability was calculated as a ratio of the averaged optical density in the wells, treated by the toxin, to the averaged optical density in the wells with the cells not treated by the toxin (control). Plotting the cytotoxicity curves and calculation of IC_{50} (targeted toxin concentration, at which the relative cell viability amounts to 50% of the control) was performed by nonlinear regression method using GrapgPad Prism program.

Confocal microscopy. To carry out the experiments for determining the binding of protein DARPin-mCherry-PE40 to HER2, cells in the quantity of 25,000 were inoculated in the glass bottom dishes (WillCo Wells, Netherlands) in 1.5 ml volume and cultured overnight at 37°C in the atmosphere of 5% CO_2. Before the experiment DARPin-mCherry-PE40 (30 nM) was added to the cells, incubated at 4°C and 37°C for 10 min, the cells were then washed 3 times by phosphate saline buffer and visualized by means of Carl Zeiss LSM-710-NLO confocal microscope (Carl Zeiss, Germany) using the following parameters: excitation by 561 nm laser, fluorescence registration in the range of 570–735 nm.

Results and Discussion. Advancement of molecular medicine dictates the necessity of developing new drugs providing highly sensitive detection and highly selective therapy of malignant neoplasms.

Previously in our laboratory hybrid protein DARPin-mCherry was designed for highly specific fluorescent visualization of the cells overexpressing HER2 receptor [18]. This recombinant protein contains a HER2-specific targeted module based on non-immunoglobulin scaffold with ankyrin repeats DARPin_9-29 [15] and an imaging module based on a fluorescent protein mCherry whose red emission spectrum [16] is in the "therapeutic transparency window" of the biotissue. By means of confocal microscopy, flow cytometry and surface plasmon resonance it was shown that DARPin-mCherry

specifically binds with HER2 oncomarker. So its can be considered is a perspective HER2-specific visualizing agent in the molecular biological researches [18]. Later we obtained and functionally characterized genetically encoded targeted DARPin-PE40 toxin [17], possessing a high selective cytotoxicity in respect to HER2-positive cells (IC_{50}=0.2 pM), in which cytotoxic module based on Pseudomonas exotoxin A fragment (40 kDa) was added to the targeted module DARPin_9-29. Cytotoxic module PE40 is a fragment of natural exotoxin A (252–613 a.a.) from *Ps. aeruginosa* containing translocation domain II and domains Ib and III of the natural toxin responsible for ADP-ribosylation of eukaryotic translation elongation factor 2. PE40 causes irreversible inhibition of protein synthesis in eukaryotic cells.

Having combined in a single protein molecule all three functions necessary for a theranostic agent — targeted, diagnostic and therapeutic — we obtained a recombinant theranostic agent DARPin-mCherry-PE40, enabling both specific visualization and elimination of HER2-positive human cancer cells.

The genetic construction for expression of recombinant protein DARPin-mCherry-PE40 in bacterial system under the control of inducible promoter T7 contains encoding sequences of the targeted module DARPin_9-29, imaging module mCherry, and cytotoxic module PE40 in one reading frame (See Figure 1 (a)). Sequences, encoding hexahistidine tag (for purification of the recombinant protein by means of metal chelate affinity chromatography), and a signaling peptide KDEL, providing translocation of the toxin from Golgi apparatus to endoplasmic reticulum, are envisaged on the 3′-terminal of the genetic construction [20].

Gene expression of the targeted protein DARPin-mCherry-PE40 was performed in *E. coli* strain BL21(DE3). DARPin-mCherry-PE40 was purified by metal chelate affinity chromatography (See “Materials and Methods”). As it is seen from the profile of elution by imidazole gradient, the protein comes off the column as a single symmetric peak (See Figure 1 (b)). Electrophoretic analysis of these peak fractions (Figure 1 (c)) showed, that the purity of the protein meets the requirements of the cell work. The protein yield was 35 mg/L culture.

The presence of a targeted module specific to HER2 oncomarker in the hybrid protein DARPin-mCherry-PE40 suggests a high interaction of the given protein with this receptor. Assessment of DARPin-mCherry-PE40 binding to the surface of SK-BR-3 tumor cells overexpressing HER2 receptor was carried out with the help of confocal microscopy. It has been estimated, that in case of incubation of HER2-positive cells with hybrid protein DARPin-mCherry-PE40 for 10 min at 4°C (conditions, excluding receptor-mediated protein internalization), effective binding of recombinant protein with a cell membrane takes place (Figure 2). Further incubation of SK-BR-3 cells, subject to the treatment by DARPin-mCherry-PE40 at 37°C for 10 min, results in internalization of hybrid protein DARPin-mCherry-PE40 into the cell, which proves active receptor-mediated endocytosis. A fluorescent signal on HER2-negative cells CHO-K1, treated by DARPin-mCherry-PE40 at 37°C could not be detected, which testifies to the specific interaction of DARPin-mCherry-PE40 with HER2.

Spectrophotometric MTT viability test of HER2-overexpressing cells SK-BR-3 in comparison with the viability of HER2-negative cells CHO-K1 was used to determine specific cytotoxicity of hybrid toxin DARPin-mCherry-PE40. Treatment of HER2-positive cells SK-BR-3 by DARPin-mCherry-PE40 was found to result in specific decrease of viability of these cells in the range of picomolar

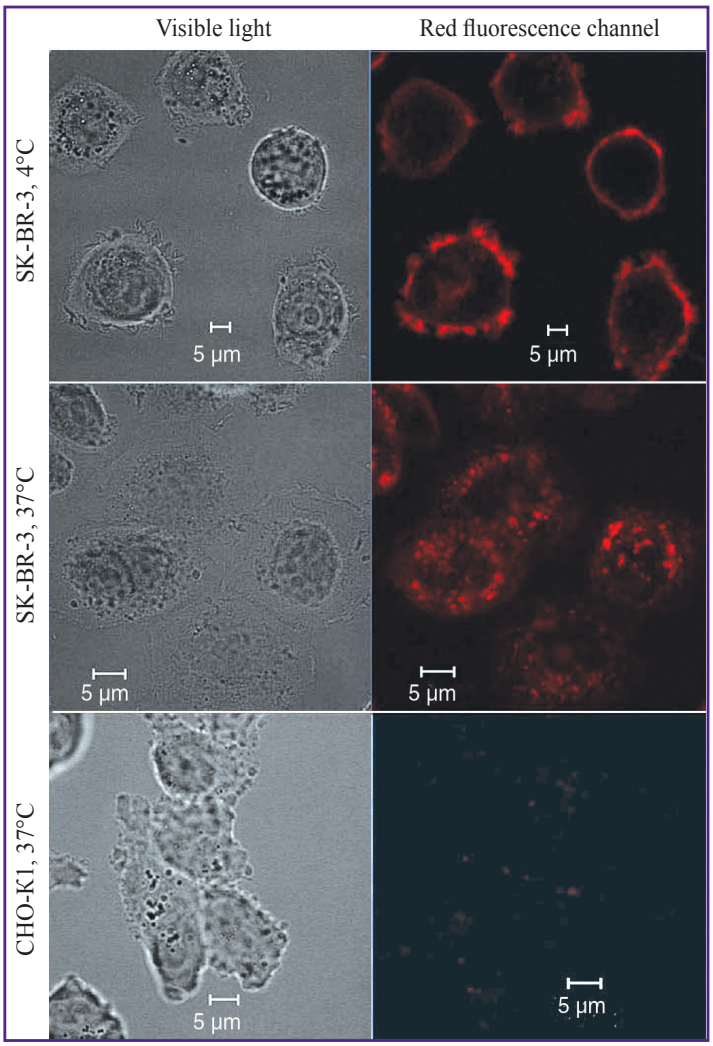

Figure 2. Interaction of hybrid protein DARPin-mCherry-PE40 with HER2. Confocal images of SK-BR-3 cells, treated by DARPin-mCherry-PE40 at 4°C, and also SK-BR-3 and CHO-K1 cells, treated by the targeted toxin at 37°C are presented. The left panel corresponds to the cell images in the visible light, the right one corresponds to the fluorescent images of the cells in the red channel

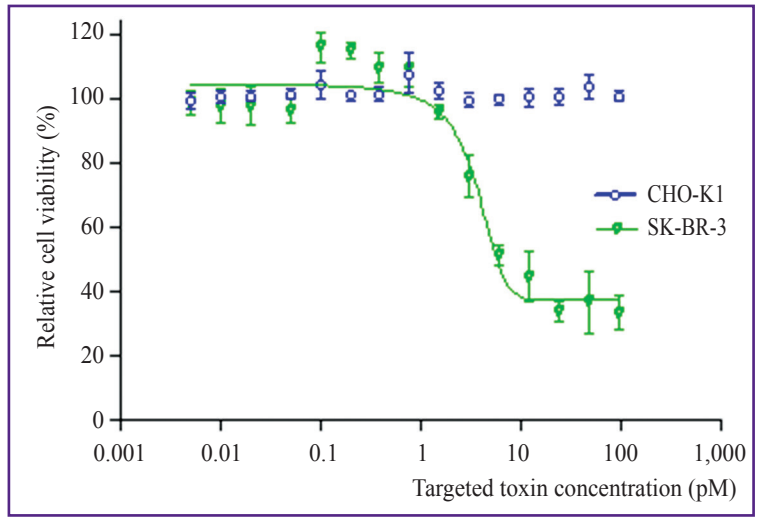

Figure 3. Analysis of DARPin-mCherry-PE40 cytotoxicity *in vitro*. Relative viability of HER2-positive cells SK-BR-3 and HER2-negative cells CHO-K1 after their treatment by targeted toxin DARPin-mCherry-PE40 for 72 h

concentrations (IC_{50}=3.55 pM) (Figure 3). However, a significant cytotoxic effect in the given concentration range of toxin DARPin-mCherry-PE40 was not observed for cells CHO-K1 not overexpressing HER2 receptor. The obtained data speak of a selective cytotoxicity of DARPin-mCherry-PE40 in respect to tumor cells overexpressing HER2 oncomarker.

Conclusion. An active recombinant targeted toxin DARPin-mCherry-PE40, obtained in soluble form during bacterial expression, is a promising theranostic agent for both detection and elimination of HER2-positive cancer cells. A high selective cytotoxicity of DARPin-mCherry-PE40 relative to HER2-positive cells, proved by confocal microscopy and spectrophotometric MTT test, testifies to the fact, that all three domains in the recombinant protein preserved their functional properties — high affinity to the tumor antigen, ability to fluorescence and cytotoxicity. Thus, the constructed multifunctional recombinant protein DARPin-mCherry-PE40 combines the functions of selective and highly effective detection of cancer cells and selective therapeutic impact on them, realizing the principle when the whole is greater than the sum of its constituent parts.

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Conflicts of Interest. The authors declare no conflicts of interest.

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