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A DUAL-VECTOR PHOSPHOLIPID NANOSYSTEM OF DOXORUBICIN: ACCUMULATION AND CYTOTOXIC EFFECT IN BREAST CANCER CELLS *IN VITRO*

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Various chemotherapeutic agents are used to treat breast cancer (BC); one of them is the anthracycline antibiotic doxorubicin (Dox), which, in addition to its cytostatic effect, has serious side effects. In order to reduce its negative impact on healthy organs and tissues and to increase its accumulation in tumors, Dox was incorporated into phospholipid nanoparticles. The additional use of vector molecules for targeted delivery to specific targets can increase the effectiveness of Dox due to higher accumulation of the active substance in the tumor tissue. The integrin $\alpha_v\beta_3$, which plays an important role in cancer angiogenesis, and the folic acid receptor, which is responsible for cell differentiation and proliferation, have been considered in this study as targets for such vector molecules. Thus, a phospholipid composition of Dox containing two vector ligands, cRGD peptide and folic acid (NPh-Dox-cRGD-Fol(3,4)), was prepared. Study of the physical properties of the developed composition NPh-Dox-cRGD-Fol(3,4) showed that the average particle size was 39.62±4.61 nm, the ζ-potential value was 4.17±0.83 mV. Almost all Dox molecules were incorporated into phospholipid nanoparticles (99.85±0.21%). The simultaneous use of two vectors in the composition led to an increase in the Dox accumulation in MDA-MB-231 BC cells by almost 20% as compared to compositions containing each vector separately (folic acid or the cRGD peptide). Moreover, the degree of Dox internalization was 22% and 24% higher than in the case of separate use of folic acid and cRGD peptide, respectively. The cytotoxic effect on MDA-MB-231 cells was higher during incubations with the compositions containing folic acid as a single vector (NPh-Dox-Fol(3,4)) and together with the RGD peptide (NPh-Dox-cRGD-Fol(3,4)). Experiments on the Wi-38 diploid fibroblast cell line have shown a significantly lower degree of cytotoxic effect of the phospholipid composition, regardless of the presence of the vector molecules in it, as compared to free Dox. The results obtained indicate the potential of using two vectors in one phospholipid composition for targeted delivery of Dox.

Key words: breast cancer; phospholipid nanoparticles; cRGD; integrin $\alpha_v\beta_3$; chemotherapy; doxorubicin; folate receptor

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INTRODUCTION

Breast cancer (BC) is the most common cancer in women. In Russia, the absolute number of BC cases in 2021 was 12.1% in the structure of the total cancer incidence [1]. In women, the BC incidence is 22.1% of the total number of all oncological pathologies and ranks first. BC is a genetic disease, consisting of different subtypes, with distinct molecular characteristics and genetic profiles: normal, luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched and basal-like [2, 3]. BC treatment includes together with surgery and radiation therapy. One widely used chemotherapy drug is doxorubicin (Dox). However, its clinical use is still limited due to cardiotoxicity and multidrug resistance (MDR) [4, 5]. In the last few decades, cancer therapy has undergone changes due to the development of nanotechnologies that makes nanoparticles (NPs) with various shapes, sizes, surface properties and controlled effect *in vitro* / *in vivo* [6]. The use of NPs as nanocarriers improves the properties (optimization) of drugs, in particular, increases bioavailability due to passive targeting of membrane structures, increased permeability and retention time, or active targeting based on ligand-receptor interaction [7, 8]. Research uses submicron-sized particles (3–200 nm) produced using various materials, including lipids (liposomes), polymers (polymer NPs, micelles, or dendrimers), viruses (viral NPs), and even organometallic compounds (nanotubes) [9, 10].

Liposomes attract much attention due to their high biocompatibility, low toxicity, high inclusivity and improved bioavailability of drugs incorporated into them [11]. Liposomes have some passive targeting ability, but the effect of passive targeting itself is very limited, while active targeting is an effective strategy to improve drug accumulation in the target tissue. Active targeting of drug-carrying liposomes has been the subject of study over the past several decades [12]. Liposomes can be modified by conjugating them with specific ligands that can selectively interact with receptors overexpressed on the surface of tumor cells. Such modifications increase the accumulation of the drug inside the tumor and enhance its therapeutic effect [13].

Characterization of the properties of the tumor and investigation of biological processes involved in its angiogenesis can play a decisive role in minimizing the BC incidence and associated mortality. In this context, integrin $\alpha_v\beta_3$, expressed on endothelial and

some other tumor cells, is of great importance in the BC angiogenesis. A tripeptide with the sequence arginine-glycine-aspartate (Arg-Gly-Asp, RGD) demonstrates preferential binding to integrin $\alpha_v\beta_3$, which is highly expressed in the new endothelial vascular system and is also capable of inducing apoptosis and tumor vessel death [14]. Thus, targeting tumor vessels or tumor cells using RGD peptide-based vectors is a promising strategy for BC therapy. Modification of Dox-containing nanoparticles with an RGD ligand is expected to improve drug transport into tumor cells [15].

Small molecules, such as folic acid (FA), are also used for targeted drug delivery. This water-soluble B vitamin, found in leafy green crops, vegetables and other plants is involved in DNA synthesis or cell division and therefore is essential for all cells. FA is transported into healthy or cancer cells via cell surface folate receptors. Folate receptor expression in cancer cells is higher than in normal cells [16]. In the context of Dox delivery to breast cancer cells, the use of FA together with graphene oxide was effective and safe *in vitro* and *in vivo* [17]. The use of FA as an independent targeting fragment for the delivery of Dox to BC cells was also effective *in vitro* and *in vivo* [18–20].

Taking into account all the above considered, the phospholipid composition with Dox, previously developed at the Institute of Biomedical Chemistry (IBMC) [21–24], was modified with two vector molecules — FA and the cyclic peptide RGD (cRGD) for the targeted delivery of Dox. Several variants of phospholipid compositions of Dox with targeted ligands (FA and cRGD) were obtained and the accumulation of Dox in tumor cells and cytotoxic effects were assessed *in vitro*.

MATERIALS AND METHODS

Preparation of Dox Nanocompositions with Target Ligands FA and cRGD

The compositions were obtained using soy phosphatidylcholine Lipoid S100 (Lipoid, Germany). Doxorubicin hydrochloride was provided by the Omutninsk Scientific Experimental Industrial Base (Russia). The DSPE-Peg2000-cRGD conjugate was prepared according to the method described in [25], using the targeting cyclic peptide cRGDfC (Synton-Lab, Russia) and the linker DSPE-Peg2000-Maleimid (Nanosoft Polymers, USA). The molar ratio of DSPE-Peg2000-Maleimid:cRGDfC was 1:2. The starting reagents were dissolved in PBS (0.01 M) (PanEko, Russia) + EDTA (2 mM) (Sigma-Aldrich, USA) + TEA (triethylamine, 2 mM) (Fluka, Belgium) (pH 7.4). The incubation mixture was bubbled with argon, incubated at room temperature and stirring for 24 h. Next, dialysis (3.5 kDa) was carried out against water for 48 h to remove unreacted substances and then lyophilized. The FA conjugate DSPE-Peg3400-Folate (Nanosoft Polymers) was used as the second vector. To prepare Dox compositions, the ratios of components indicated in Table 1 were used.

The composition with targeted ligands was obtained using the "film method" similar to the method described in [26]. Weighed portions of Lipoid S100, DSPE-Peg2000-cRGD, and DSPE-Peg3400-Folate were dissolved in a small amount (2–3 ml) of ethyl alcohol (Medkhimprom, Russia). The resulting alcohol solutions were mixed. The alcohol was then evaporated on a Heidolph Laborota 4003 rotary evaporator (Heidolph, Germany) for 8–10 min under the following conditions: 60 mbar, water temperature in the bath 30°C, rotor speed 1190 rpm. The resulting lipid film was rehydrated with distilled water with the addition of Dox. The rough emulsion was treated on a Bandelin Sonopuls ultrasonic disintegrator (Bandelin, Germany) using a KE72 titanium rod for 6 min at 50% power. Comparison samples were prepared similarly.

In the resulting Dox-containing compositions, the particle size and ζ-potential were determined using a Zetasizer Nano ZS analyzer (Malvern, UK) with Malvern ZETASIZER 6.20 software. The percentage of Dox incorporation into NPs was assessed by ultrafiltration using VivaSpin 500 microfilters (Sartorius AG, Germany). The Dox concentration was determined by HPLC using an Agilent 1100 Series chromatographic system (Agilent Technologies, USA) [27].

Stability Assessment of the Dox Compositions

The stability of the resulting compositions was studied in distilled water, PBS (pH 7.4), and DMEM medium (PanEco), after diluting the compositions 10-fold with the appropriate solution. Stability was assessed by changes in particle size in solution at certain time intervals (0 h; 0.25 h; 0.5 h; 1 h; 3 h; 24 h; and 48 h). The particle size was measured using a Zetasizer Nano ZS analyzer.

Table 1. The ratio of main components for preparation of the phospholipid compositions of Dox (w/w)

Components / Compositions	Lipoid S100	Dox	DSPE-Peg2000-cRGD	DSPE-Peg3400-Folate
NPh-Dox	20	1	—	—
NPh-Dox-cRGD	20	1	1	—
NPh-Dox-Fol(3,4)	20	1	—	1
NPh-Dox-cRGD-Fol(3,4)	20	1	1	1

The change in the percentage of Dox incorporation into NPs was monitored after 1 h and 24 h using VivaSpin 500 microfilters and an Agilent 1100 Series chromatography system.

Cell Cultures

The experiment used cell lines of triple-negative BC MDA-MB-231, cervical cancer HeLa and diploid human fibroblast cell line Wi-38. All cell lines obtained from the American Type Culture Collection (ATCC) were maintained in the IBMC cell culture collection. Cultivation of MDA-MB-231, HeLa, and Wi-38 cells was carried out according to the recommendations in the ATCC cell culture certificate. For this purpose, the necessary media were used with the addition of 10% fetal calf serum (PanEco). MDA-MB-231, HeLa, and Wi-38 cells were cultured at 37°C in an atmosphere with a relative humidity of 95% containing 5% CO_2 (a CO_2 incubator "Sanyo", Japan). In this work, cell lines from 3 to 10 passages were used.

Cell Binding and Penetration Assessment

The cell cultures MDA-MB-231, HeLa, and Wi-38 (10^6 cells per well) were seeded into 6-well culture plates (Biologix, China) and incubated for 24 h at 37°C. Free Dox was used as a control. Samples of the obtained compositions and free Dox were added at a concentration of 14 μg/ml (in terms of Dox) and incubated for 3 h at two temperature conditions: 37°C in a CO_2 incubator (Sanyo, Japan) and 4°C in a refrigerator (Atlant, Belarus). Next, the medium with the compositions was removed, and the cells were washed 2 times with PBS. Dox extraction was performed with acetonitrile solution (Fisher Scientific, UK) with the addition of 0.1% formic acid (Sigma, USA) (1 ml per well). The collected extracts were separated by centrifugation at 10,000 rpm for 10 min on an Eppendorf 5810R centrifuge (Eppendorf, Germany). The Dox concentration in the obtained samples was measured using an Agilent 1200 Series HPLC system with an Eclipse XDB-C18 column (Agilent Technologies) and a 6130 Quadrupole LC/MS mass spectrometric detector (Agilent Technologies) [24]. The Dox content in cell cultures was normalized to the protein content (mg), which was determined by the Lowry method.

Internalization was calculated by the difference in Dox content at 37°C (total accumulation in cells) and at 4°C (attachment to the cell surface) [28].

The Cytotoxic Effect In Vitro

The cytotoxic effect of the developed phospholipid composition Dox with two targeted molecules RGD and FA (NPh-Dox-cRGD-Fol(3,4)) was assessed on the triple-negative BC cell line MDA-MB-231, HeLa cervical cancer, and a diploid human fibroblast cell line Wi-38. Free Dox incorporated in phospholipid NPs (NPh-Dox), and in phospholipid NPs with targeted cRGD peptide (NPh-Dox-cRGD) and also Dox incorporated in phospholipid NPs with a folate conjugate (NPh-Dox-Fol(3,4)) were used as reference drugs for comparison of the effect of the phospholipid composition containing Dox with two targeted molecules.

MDA-MB-231, HeLa, and Wi-38 cells (7.5×10^3 cells per well) were seeded into sterile 96-well culture plates and incubated at 37°C in an atmosphere of 5% CO_2 for 24–26 h. Then the test substances/compositions with the following Dox concentrations were added: 0.025 μg/ml; 0.05 μg/ml; 0.5 μg/ml; 2.5 μg/ml; 5 μg/ml; 7.5 μg/ml; and 15 μg/ml. Cells were incubated for 24 h and 48 h.

After this, 60 μl of MTT (1 mg/ml) was carefully added to each well and incubated at 37°C for 3 h. Then the medium was removed and 100 μl of DMSO (PanEco) was added. Plates were covered with foil and shaken on an orbital shaker for 15 min. Absorbance was recorded at 570 nm (Multiscan FC, ThermoSpectronic, USA) and normalized to the untreated control (without Dox).

Cell viability was calculated using the following equation (1):

$$\text{Cell viability (\%)} = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\% \quad (1).$$

Statistical Processing

To assess the significance of differences in measured parameters (in three replicates) the Student's *t* test was used. Differences were considered statistically significant at $p \leq 0.05$. In the figures, data are presented as mean ± standard error of the mean.

RESULTS AND DISCUSSION

The development of effective drugs for the BC treatment is an important direction. For the selective accumulation of a drug in a tumor, its inclusion in transport systems containing a targeted component is of particular interest. Various substances that have an affinity for receptors overexpressed on the surface of tumor cells can act as target molecules [29–32]. In this context, the overexpression of integrin $\alpha_v\beta_3$ and folate receptor (FR) on the surface of tumor cells [29, 30] is most interesting. In this work, the simultaneous incorporation of two vectors into a phospholipid Dox-containing composition was carried out in order to increase the accumulation of the drug in BC cells. The cRGD peptide was used as a targeting component for integrin $\alpha_v\beta_3$; FA in the form of a pegylated conjugate was used for FR. The properties of the obtained Dox composition with two targeted components (NPh-Dox-cRGD-Fol(3,4)) were assessed in comparison with compositions without targeted molecules (NPh-Dox) and with

variants of each targeted molecule separately (NPh-Dox-cRGD, NPh-Dox-Fol(3,4)). Table 2 shows the results of the study of the physicochemical properties of the obtained compositions.

All obtained compositions were relatively uniform in particle size. The additional incorporation of the targeted component contributed to the particle size enlargement. According to the literature [33], particles ranging in size from 2 nm to 200 nm have been shown in many studies to have a higher accumulation rate in the tumor, since they are not recognized by the reticuloendothelial system (RES) and are not filtered by the kidneys [33]. The particle size in the studied phospholipid composition with two targeting agents was 39.62±4.61 nm. The introduction of a targeting peptide ligand led to a twofold increase in the particle size, unlike the folate vector, which had no effect on this parameter.

Another important parameter for characterization of the properties of nanosystems is the ζ-potential, which characterizes the stability of the resulting compositions. The following classification of NP dispersions according to the ζ-potential value exists in the literature: ±0–10 mV (unstable), ±10–20 mV (relatively stable), ±20–30 mV (moderately stable), and > ±30 mV (highly stable) [34]. ζ-potential measurements provide an accurate analysis of the electronic state of the NP surface, and the data obtained can be used to predict the stability of formulations containing these NPs. Instability can result from interactions between weakly charged or uncharged NPs, leading to the aggregate formation [35, 36]. A study of the ζ-potential values of the developed compositions showed low values for all variants — less than 10 mV, thus indicating their instability over a long time. Therefore, all necessary experiments must be carried out within 24 h after preparing the samples. Accordingly, to obtain the finished forms of the drug it is necessary to use cryoprotectors followed by lyophilization.

The degree of drug incorporation into phospholipid NPs, as one of the important characteristics of transport nanosystems, was assessed using the ultrafiltration method. In all samples of phospholipid compositions, Dox was almost completely incorporated into the NPs; the inclusion rate was at least 99% (Table 2). Since similar data have been obtained in our previous studies of all the developed compositions [26, 27, 37], this indicates the effectiveness of the chosen method and production conditions.

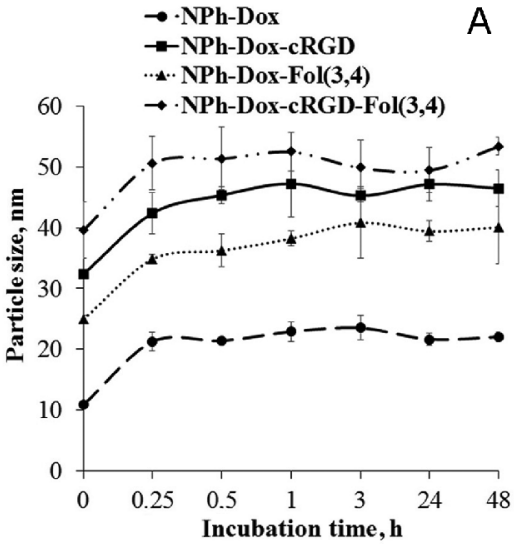
Since their main disadvantage of nanoemulsions is agglomeration of NPs and release of drugs from them the stability of nanoemulsions characterizes preservation of the aggregate state of the system during storage [38]. Therefore, stability assessment is the main step in checking the properties of developed nanocompositions. In order to establish the preferential solvent *in vitro* we have assessed changes in the particle size, polydispersity index (PdI) during incubation (25±3°C) in various media (water, phosphate-buffered saline and DMEM cell incubation medium). Figure 1 shows data on particle size changes.

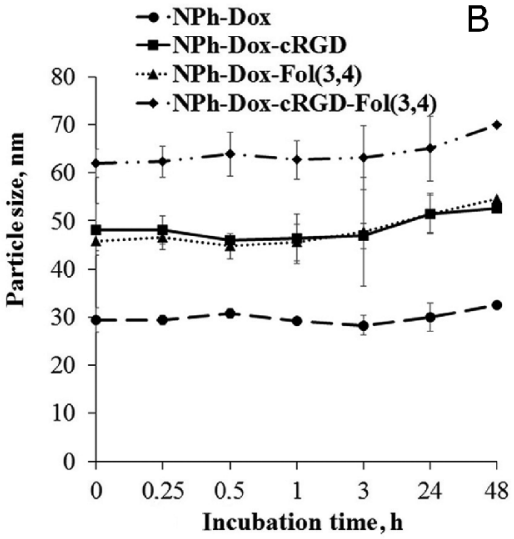
Studies of particle size changes showed that in water (Fig. 1A) the particle size increased by 10 nm in all samples after 15 min of incubation. No significant particle size changes were observed during incubation in PBS (Fig. 1B) or in DMEM medium (Fig. 1C); however, at the starting point (immediately after dilution) the particle size was already almost 1.5 times higher than the values for the corresponding compositions. In these solvents (PBS and DMEM), particles initially became larger, possibly due to their fusion. After 24 h, an increase in the particle size by 5 nm and 10 nm was noted for the NPh-Dox composition (Fig. 1B), and for NPh-Dox-cRGD and NPh-Dox-Fol(3,4), respectively.

In DMEM medium (Fig. 2B), a high degree of stability was observed (as PdI values) during 48 h in samples with targeted fragments. The particles were more homogeneous: the polydispersity index was within 0.300. For the phospholipid composition (NPh-Dox), a fairly high PdI value was observed. This suggests the presence of particles of a different size, but their percentage in the total volume was quite small. At the same time, in the buffer solution (PBS) (Fig. 2B), an increase in PdI values was noted for compositions with address vectors (NPh-Dox-cRGD, NPh-Dox-Fol(3,4), and NPh-Dox-cRGD-Fol(3,4)) after 48 h thus indicating a change in the composition of the particles: the distribution became wider (heterogeneous). In water (Fig. 2A), there was an almost similar pattern, showing that particles with the targeted fragment were more stable. For the phospholipid composition without targeting ligands, an increase in the PdI value was observed after 24 h. Previously, in order to stabilize the phospholipid composition NPh-Dox a cryoprotector was introduced and the system was then lyophilized [27].

Table 2. Physico-chemical properties of phospholipid Dox-containing compositions

Parameters \ Samples	NPh-Dox	NPh-Dox-cRGD	NPh-Dox-Fol(3,4)	NPh-Dox-cRGD-Fol(3,4)
Particle size, nm (% of particles)	20.80±0.92 (99.87±0.1%)	44.68±0.93 (100±0%)	24.73±0.79 (100±0%)	39.62±4.61 (99.85±0.21%)
ζ-potential, mV	8.50±0.12	7.66±0.35	9.11±0.34	4.17±0.83
Dox inclusion percentage in NPs	99.9±0.14	99.65±0.49	99.51±0.15	99.85±0.21





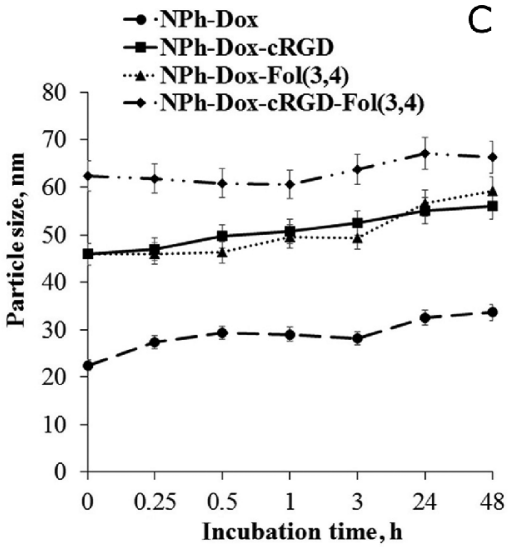
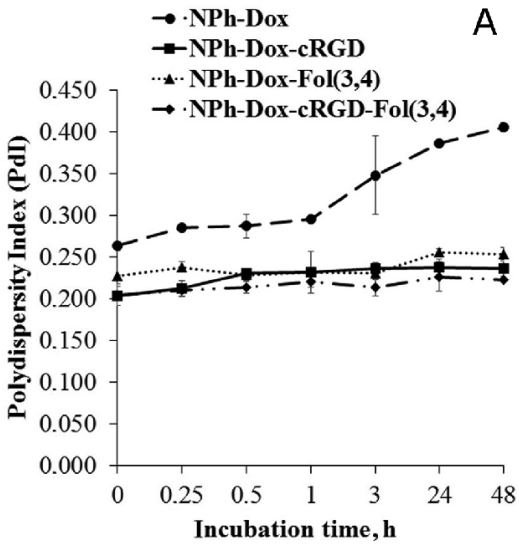
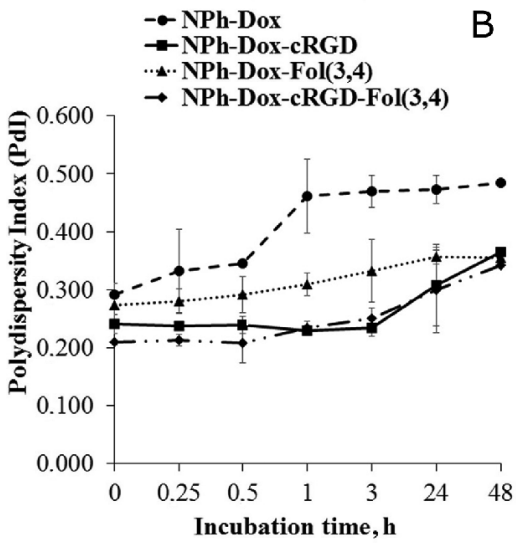


Figure 1. The time dependence of the particle size of the obtained compositions NPh-Dox, NPh-Dox-cRGD, NPh-Dox-Fol(3,4), and NPh-Dox-cRGD-Fol(3,4) incubated at 25±3°C in water (**A**), PBS (**B**), and DMEM (**C**) (n=3).





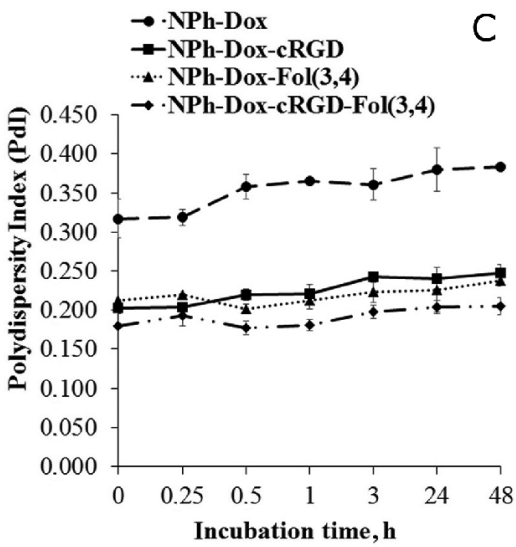


Figure 2. The time dependence of the polydispersity index (PdI) of the obtained compositions NPh-Dox, NPh-Dox-cRGD, NPh-Dox-Fol(3,4), and NPh-Dox-cRGD-Fol(3,4), incubated at 25±3°C in water (**A**), PBS (**B**), and DMEM (**C**) (n=3).

The results of evaluation of the percentage of Dox incorporation into NPs after incubation for 1 h and 24 h are given in Table 3.

After 24 h-incubation in water, a decrease in the percentage of Dox incorporated in the NPs was noted. However, for the composition containing two address vectors, the percentage of inclusion decreased to a lesser extent as compared to other samples and amounted to more than 90%. In the case of incubation in PBS, a decrease in this parameter was also observed, similar to the incubation in water. After 24 h, the inclusion percentage was not lower than 91%. A study of stability in DMEM showed its high degree during storage for 24 h.

Accumulation of the developed composition in cells, was evaluated using BC cell line (MDA-MB-231) expressing the surface integrin $\alpha_v\beta_3$ and FR [39, 40]. In these experiments HeLa and Wi-38 cell lines were used as controls. The HeLa cell line expresses FR but it is integrin $\alpha_v\beta_3$ negative. On the contrary, the Wi-38 cell line, is FR-negative, but is integrin $\alpha_v\beta_3$ positive [41–43].

The study on the MDA-MB-231 cell culture (Fig. 3A) showed some dependence on the presence of a targeting ligand in the Dox-containing phospholipid nanosystem. The maximum values of total Dox accumulation were observed in the case of the composition with two vectors (1.26 μg/mg protein), exceeding the values with each individual vector (Fol and cRGD) by 19.8% and 12.7%, respectively. In turn, the values for the given composition with two vectors were different depending on the cell line used. For example, the total accumulation in Wi-38 cells was 0.88 μg/mg of protein, while in the case of HeLa cells it was 0.48 μg/mg of protein.

For the HeLa cell line (Fig. 3B), results for total accumulation showed a 2-fold increase in Dox accumulation in the case of the use of the folate vector as compared to free Dox.

According to the literature data, samples containing the RGD peptide should act on the control line Wi-38 (Fig. 3B). The results of the total accumulation data showed that the composition with the peptide vector showed less influence as compared to the two-vector composition (NPh-Dox-cRGD-Fol(3,4)). Moreover, the folate vector in the composition NPh-Dox-Fol(3,4) showed a higher effect on this cell line along with free Dox.

It should be noted that the expected effect was not obtained on the control lines. However, on the BC cells expressing both receptors (integrin and FR), the targeted effect of a phospholipid composition with two vectors, was recognized primarily due to a high degree of Dox internalization into the cell (Fig. 3A).

Figure 4 shows results of the study of the cytotoxic effect of the developed composition NPh-Dox-cRGD-Fol(3,4). Incubation of triple-negative BC cells MDA-MB-231 with the developed Dox compositions for 24 h showed that at concentrations of 5–15 μg/ml, the compositions NPh-Dox-Fol(3,4) and NPh-Dox-cRGD-Fol(3,4) had a more pronounced cytotoxic effect (Fig. 4A). At the same time, the percentage of tumor cell death in the variant of incubation with the compositions NPh-Dox-Fol(3,4) and NPh-Dox-cRGD-Fol(3,4) (with a Dox concentration of 15 μg/ml) was 1.7 times higher as compared to free substance (Dox). After 48 h-incubation with the substances, the same dependence on the concentration of the active

Table 3. Changing the Dox inclusion percentage in NPs during incubation in different media

Samples	Incubation time, h		
	0	1	24
H_2O			
NPh-Dox	95.90±0.13	84.33±0.39	70.10±0.55
NPh-Dox-cRGD	99.80±0.19	95.91±0.21	86.13±0.34
NPh-Dox-Fol(3,4)	99.50±0.23	92.17±0.13	83.00±0.17
NPh-Dox-cRGD-Fol(3,4)	99.75±0.09	97.00±0.49	91.01±0.15
PBS			
NPh-Dox	95.90±0.33	91.41±0.34	91.27±0.16
NPh-Dox-cRGD	98.12±0.11	93.00±0.14	94.00±0.44
NPh-Dox-Fol(3,4)	97.00±0.25	95.45±0.37	94.87±0.11
NPh-Dox-cRGD-Fol(3,4)	99.23±0.15	98.01±0.09	97.33±0.60
DMEM			
NPh-Dox	99.00±0.22	99.11±0.19	99.78±0.09
NPh-Dox-cRGD	99.87±0.04	99.17±0.70	99.89±0.07
NPh-Dox-Fol(3,4)	99.82±0.05	99.62±0.10	99.89±0.05
NPh-Dox-cRGD-Fol(3,4)	99.53±0.08	99.10±0.09	99.91±0.05

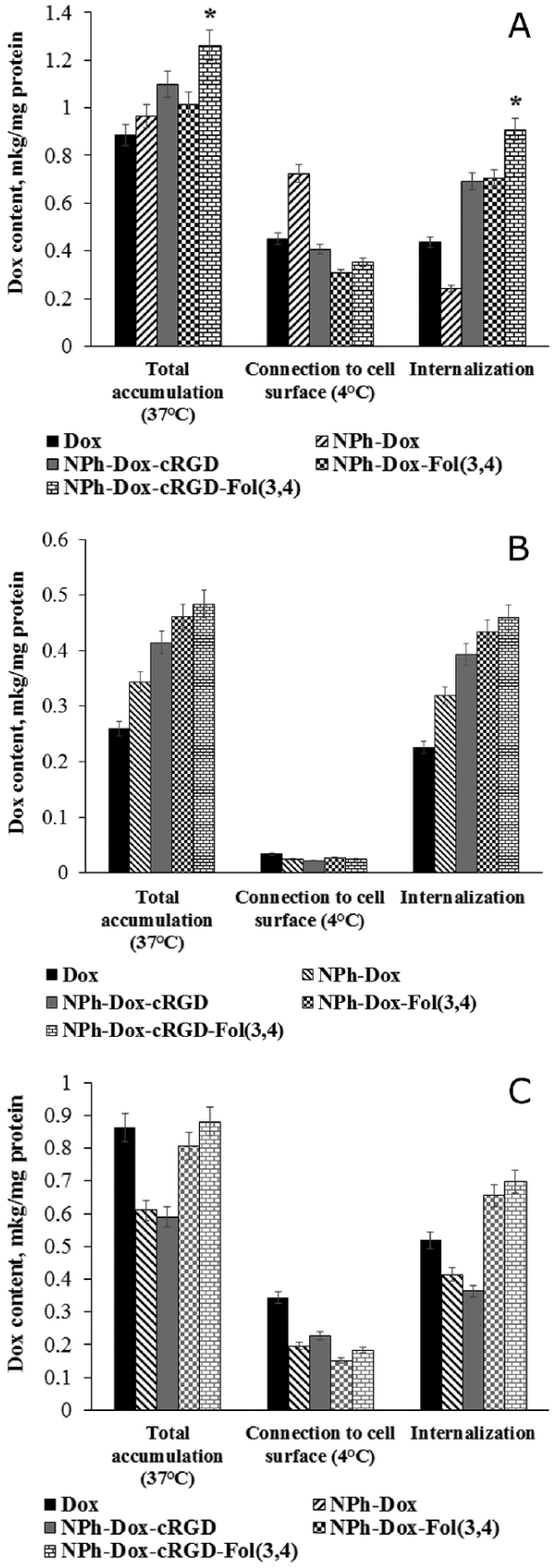


Figure 3. Dox accumulation in MDA-MB-231 (**A**), HeLa (**B**), and Wi-38 (**C**) cell cultures after 3 h incubation with the obtained compositions and free Dox (n=3).

substance in the composition was observed (almost the same degree of the effect on cell death at 0.025 μg/ml and 0.05 μg/ml) (Fig. 4B); however, between samples of the same concentration no statistically significant differences were noted.

For the control HeLa cell line, the percentage of cell viability was higher than for MDA-MB-231 cells both after 24 h- and 48 h-incubation (Fig. 4C and 4D). After 48 h-incubation at 0.025 μg/ml, no significant differences in the percentage of viability were found between the studied samples; after 24 h-incubation in the variant with the phospholipid composition (NPh-Dox), this parameter at the concentrations of 0.025 μg/ml and 0.05 μg/ml exceeded the value of free Dox substance by 7% and by 12%, respectively.

According to the literature data [14], the targeting RGD peptide selectively binds to integrin $\alpha_v\beta_3$. In this regard, it would be logical to expect slightly different values for compositions with this ligand (NPh-Dox-cRGD and NPh-Dox-cRGD-Fol(3,4)). However, in practice, no significant differences between the samples of phospholipid compositions were recorded, nor any concentration dependence (Fig. 4D and 4E). Only in the case of the concentration of 7.5 μg/ml the incubation with the NPh-Dox-cRGD composition for 48 h showed more than 2-fold higher value in cell viability as compared with the composition containing two vectors. In other words, at this concentration the degree of cytotoxic effect on healthy cells for this sample was minimal. The effect of free Dox on the diploid human fibroblast cell line Wi-38 was significantly more negative after 24 h-incubation; at the minimum concentration (0.025 μg/ml Dox) there were fewer living cells on average by almost 15% as compared to phospholipid samples. With increasing concentration of the active substance, as well as with increasing incubation time, the level of cell viability decreased. After 48 h-incubation, the percentage of living cells decreased sharply with increasing concentration as compared to phospholipid compositions. It is possible that the lesser cytostatic effect on Wi-38 cells is due to Dox incorporation into phospholipid NPs. Thus, the results obtained indicate that additional incorporation of targeted components into phospholipid NPs with Dox did not reduce its cytostatic effect on tumor cells (MDA-MB-231 and HeLa); however, in relation to healthy cells, a lesser toxic effect of phospholipid compositions was noted.

The data obtained indicate the potential of studying the simultaneous use of two targeted ligands in one composition to increase the effectiveness and safety of Dox in tumor therapy. At the same time, it is important to perform additional studies of the properties of the obtained composition after modifying some parameters: the length of the linker in the conjugate with FA for incorporation into phospholipid NPs, the ratio of the main components, and changing the incubation time of cells with

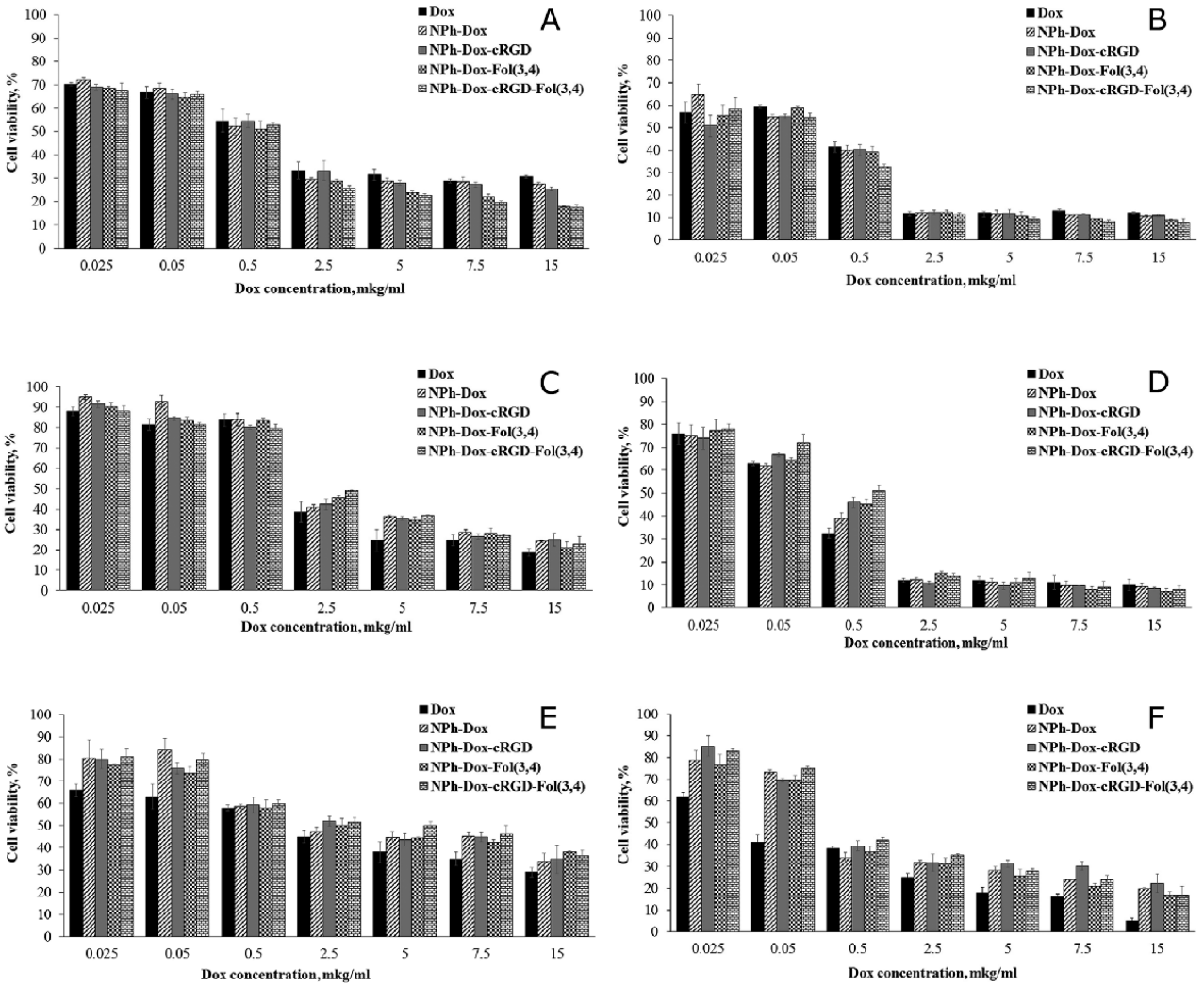


Figure 4. Viability of MDA-MB-231 (**A**, **B**) HeLa (**C**, **D**), and Wi-38 (**E**, **F**) cells after incubation for 24 h (**A**, **C**, **E**) and 48 h (**B**, **D**, **F**) with the obtained compositions and free Dox in various concentrations (n=3).

the studied substances. These studies will provide detailed information on the therapeutic potential of the developed two-vector Dox containing phospholipid composition.

CONCLUSIONS

To increase the Dox effectiveness as well as reduce MDR and reduce Dox toxicity, systems for Dox delivery directly to tumor cells are being developed. For these purposes, various vector compounds are used that target receptors (proteins) expressed on the surface of the tumor or tumor vessels. To increase the possibility of increased accumulation in the tumor area, two- and three-vector drug delivery systems are used. Our work proposes a two-vector phospholipid system to increase the Dox accumulation in BC cells. The obtained composition represents an ultrathin emulsion with a particle size of up to 100 nm in which almost all Dox (at least 98%) is incorporated into the NPs. Stability analysis showed that the formulations were stable for 48 h in media, with little changes observed in water and PBS at 48 h. The overall accumulation and internalization of Dox on MDA-MB-231 cells increased by ~1.4 and ~1.3 times when two targeting vectors were used (in comparison to monovector compositions (NPh-Dox-cRGD and NPh-Dox-Fol(3,4), respectively). Evaluation of the cytotoxic effect showed greater death of MDA-MB-231 BC cells incubated with the compositions NPh-Dox-Fol(3,4) and NPh-Dox-cRGD-Fol(3,4). However, no pronounced cytotoxic effect was observed on the control HeLa cell line. For phospholipid compositions, regardless of the presence of targeting molecules, a lesser cytotoxic effect was shown on normal Wi-38 cells as compared to the free Dox. According to the results obtained in this study, the use of two vectors is promising for the BC treatment. The interpretation of the results obtained will be more complete after a series of additional experiments, including studies of the level of expression of selected receptors on the surface of tumor cells, the results of which will be presented in our future publications.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. *Kaprin A.D., Starinsky V.V., Shakhzadova A.O.* (2022) Malignant neoplasms in Russia in 2021 (morbidity and mortality). Moscow: P.A. Herzen Research Medical Institute — branch of the Federal State Budgetary Institution "Research Institute of Radiology" of the Ministry of Health of Russia, 252 p.
2. *Perou C.M., Sørlie T., Eisen M.B., van de Rijn M., Jeffrey S.S., Rees C.A., Botstein D.* (2000) Molecular portraits of human breast tumours. Nature, **406**(6797), 747-752. DOI: 10.1038/35021093
3. *Sorolla A., Sorolla M.A., Wang E., Ceña V.* (2020) Peptides, proteins and nanotechnology: A promising synergy for breast cancer targeting and treatment. Expert Opin. Drug Deliv., **17**(11), 1597-1613. DOI: 10.1080/17425247.2020.181473
4. *Govender J., Loos B., Marais E., Engelbrech A.-M.* (2014) Mitochondrial catastrophe during doxorubicin-induced cardiotoxicity: A review of the protective role of melatonin. J. Pineal Res., **57**, 367-380. DOI: 10.1111/jpi.12176
5. *Minko T., Rodriguez-Rodriguez L., Pozharov V.* (2013) Nanotechnology approaches for personalized treatment of multidrug resistant cancers. Adv. Drug Deliv. Rev., **65**(13-14), 1880-1895. DOI: 10.1016/j.addr.2013.09.017
6. *Dobson J.* (2006) Magnetic nanoparticles for drug delivery. Drug Devel. Res., **67**(1), 55-60. DOI: 10.1002/ddr.20067
7. *Sun T., Zhang Y.S., Pang B., Hyun D.C., Yang M., Xia Y.* (2014) Engineered nanoparticles for drug delivery in cancer therapy. Angewandte Chemie International Education England Novel, **53**(46), 12320-12364. DOI: 10.1002/anie.201403036
8. *Aghebati-Maleki A., Dolati S., Ahmadi M., Baghbanzhadeh A., Asadi M., Fotouhi A., Yousefi M., Aghebati-Maleki L.* (2020) Nanoparticles and cancer therapy: Perspectives for application of nanoparticles in the treatment of cancers. J. Cell Physiol., **235**(3), 1962-1972. DOI: 10.1002/jcp.29126
9. *Dadwal A., Baldi A., Kumar Narang R.* (2018) Nanoparticles as carriers for drug delivery in cancer. Artif. Cells Nanomed. Biotechnol., **46**(sup2), 295-305. DOI: 10.1080/21691401.2018.1457039
10. *Shafei A., El-Bakly W., Sobhy A., Wagdy O., Reda A., Aboelenin O., Marzouk A., El Habak K., Mostafa R., Ali M.A., Ellithy M.* (2017) A review on the efficacy and toxicity of different doxorubicin nanoparticles for targeted therapy in metastatic breast cancer. Biomed. Pharmacother., **95**, 1209-1218. DOI: 10.1016/j.biopha.2017.09.059
11. *Wang J., Gong J., Wei Z.* (2021) Strategies for liposome drug delivery systems to improve tumor treatment efficacy. AAPS PharmSciTech, **23**(1), 27. DOI: 10.1208/s12249-021-02179-4
12. *Zhang M., Lou C., Cao A.* (2022) Progresses on active targeting liposome drug delivery systems for tumor therapy. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi, **39**(3), 633-638. DOI: 10.7507/1001-5515.202110067
13. *d'Avanzo N., Torrieri G., Figueiredo P., Celia C., Paolino D., Correia A., Moslova K., Teesalu T., Fresta M., Santos H.A.* (2021) LinTT1 peptide-functionalized liposomes for targeted breast cancer therapy. Int. J. Pharm., **597**, 120346. DOI: 10.1016/j.ijpharm.2021.120346
14. *Ge L., You X., Huang K., Kang Y., Chen Y., Zhu Y., Ren Y., Zhang Y., Wu J., Qian H.* (2017) Screening of novel RGD peptides to modify nanoparticles for targeted cancer therapy. Biomaterials Science, **6**(1), 125-135. DOI: 10.1039/c7bm00776k
15. *Sun Y., Kang C., Liu F., Zhou Y., Luo L., Qiao H.* (2017) RGD peptide-based target drug delivery of doxorubicin nanomedicine. Drug Devel. Res., **78**(6), 283-291. DOI: 10.1002/ddr.21399
16. *Wu B., Zhao N.* (2016) A targeted nanoprobe based on carbon nanotubes – natural biopolymer chitosan composites. Nanomaterials, **6**, 216. DOI: 10.3390/nano6110216
17. *Fong Y.T., Chen C.H., Chen J.P.* (2017) Intratumoral delivery of doxorubicin on folate-conjugated graphene oxide by *in-situ* forming thermo-sensitive hydrogel for breast cancer therapy. Nanomaterials (Basel), **7**(11), 388. DOI: 10.3390/nano7110388
18. *Cé R., Couto G.K., Pacheco B.Z., Dallemole D.R., Paschoal J.D., Pacheco B.S., Guterres S.S., Seixas F., Collares T., Pohlmann A.R.* (2021) Folic acid-doxorubicin polymeric nanocapsules: A promising formulation for the treatment of triple-negative breast cancer. Eur. J. Pharm. Sci., **165**, 105943. DOI: 10.1016/j.ejps.2021.105943
19. *Kayani Z., Bordbar A.K., Firuzi O.* (2018) Novel folic acid-conjugated doxorubicin loaded β-lactoglobulin nanoparticles induce apoptosis in breast cancer cells. Biomed. Pharmacother., **107**, 945-956. DOI: 10.1016/j.biopha.2018.08.047
20. *Lale S.V., Kumar A., Prasad S., Bharti A.C., Koul V.* (2015) Folic acid and trastuzumab functionalized redox responsive polymersomes for intracellular doxorubicin delivery in breast cancer. Biomacromolecules, **16**(6), 1736-1752. DOI: 10.1021/acs.biomac.5b00244
21. *Nemtsova E.R., Tikhonova E.G., Bezborodova O.A., Pankratov A.A., Venediktova J.B., Korotkevich E.I., Kostryukova L.V., Tereshkina J.A.* (2020) Preclinical study of pharmacological properties of doxorubicin-NPh. Bulletin of Experimental Biology and Medicine, **169**, 778-782. DOI:10.1007/s10517-020-04977-5
22. *Medvedeva N.V., Torkhovskaya T.I., Kostryukova L.V., Zakharova T.S., Kudinov V.A., Kasatkina E.O., Prozorovskiy V.N., Ipatova O.M.* (2017) Influence of doxorubicin inclusion into phospholipid nanoparticles on tumor accumulation and specific activity. Biomeditsinskaya Khimiya, **63**(1), 56-61. DOI: 10.18097/PBMC20176301056

23. *Zykova M.G., Medvedeva N.V., Torkhovskava T.I., Tikhonova E.G., Prozorovskii V.N., Zakharova T.S., Ipatova O.M.* (2012) Influence of doxorubicin inclusion into phospholipid nanoformulation on its antitumor activity in mice: Increased efficiency for resistant tumor model. Experimental Oncology, **34**, 323-326.
24. *Zykova M.G, Ipatova O.M., Prozorovskii V.N., Medvedeva N.V., Voskresenskaya A.A., Zakharova T.S., Torkhovskaya T.I.* (2011) Changes in the distribution of doxorubicin in blood and plasma when it is included in the phospholipid nanocomposition. Biomeditsinskaya Khimiya, **57**(2), 174-179. DOI: 10.18097/PBMC20115702174
25. *Song Z., Lin Y., Zhang X., Feng C., Lu Y., Gao Y., Dong C.* (2017) Cyclic RGD peptide-modified liposomal drug delivery system for targeted oral apatinib administration: enhanced cellular uptake and improved therapeutic effects. Int. J. Nanomed., **12**, 1941-1958. DOI: 10.2147/IJN.S125573
26. *Kostryukova L.V., Tereshkina Y.A., Korotkevich E.I., Prozorovsky V.N., Torkhovskaya T.I., Morozevich G.E., Toropygin I.Y., Konstantinov M.A., Tikhonova E.G.* (2020) Targeted drug delivery system for doxorubicin based on a specific peptide and phospholipid nanoparticles. Biomeditsinskaya Khimiya, **66**(6), 464-468. DOI: 10.18097/PBMC20206606464
27. *Tikhonova E.G., Sanzhakov M.A., Tereshkina Yu.A., Kostryukova L.V., Khudoklinova Yu.Yu., Orlova N.A., Bobrova D.V., Ipatova O.M.* (2022) Drug transport system based on phospholipid nanoparticles: Production technology and characteristics, Pharmaceutics, **14**(11), 2522. DOI: 10.3390/pharmaceutics14112522
28. *Sheldon K., Liu D., Ferguson J., Gariepy J.* (1995) Loligomers: Design of *de novo* peptide-based intracellular vehicles. Proc. Natl. Acad. Sci. USA, **92**(6), 2056-2060. DOI: 10.1073/pnas.92.6.2056
29. *Farran B., Montenegro R.C., Kasa P., Pavitra E., Huh Y.S., Han Y.K., Kamal M.A., Nagaraju G.P., Rama Raju G.S.* (2020) Folate-conjugated nanovehicles: Strategies for cancer therapy. Mater. Sci. Eng. C, **107**, 110341. DOI: 10.1016/j.msec.2019.110341
30. *Cheng T.M., Chang W.J., Chu H.Y., de Luca R., Pedersen J.Z., Incerpi S., Li Z.L., Shih Y.J., Lin H.Y., Wang K., Whang-Peng J.* (2021) Nano-strategies targeting the integrin αvβ3 network for cancer therapy. Cells, **10**(7), 1684. DOI: 10.3390/cells10071684
31. *Li R., Peng Y., Pu Y., Zhao Y., Nie R., Guo L., Wu Y.* (2022) Fructose and biotin co-modified liposomes for dual-targeting breast cancer. J. Liposome Res., **32**(2), 119-128. DOI: 10.1080/08982104.2021.1894171
32. *Janani S.K., Dhanabal S.P., Sureshkumar R., Nikitha Upadhyayula S.S.* (2022) Anti-nucleolin aptamer as a boom in rehabilitation of breast cancer. Curr. Pharm. Des., **28**(38), 3114-3126. DOI: 10.2174/1381612828666220928105044
33. *Yetisgin A.A., Cetinel S., Zuvin M., Kosar A., Kutlu O.* (2020) Therapeutic nanoparticles and their targeted delivery applications. Molecules, **25**(9), 2193. DOI: 10.3390/molecules25092193
34. *Bhattacharjee S.* (2016) DLS and zeta potential — What they are and what they are not? J. Control. Release, **235**, 337-351. DOI: 10.1016/j.jconrel.2016.06.017
35. *Manaia E.B., Abuçafy M.P., Chiari-Andréo B.G., Silva B.L., Oshiro Junior J.A., Chiavacci L.A.* (2017) Physicochemical characterization of drug nanocarriers. Int. J. Nanomed., **12**, 4991-5011. DOI: 10.2147/IJN.S133832
36. *Xian H.W., Sidik N.A.C., Saidur R.* (2020) Impact of different surfactants and ultrasonication time on the stability and thermophysical properties of hybrid nanofluids. Int. Commun. Heat Mass Transf., **110**, 104389. DOI: 10.1016/j.icheatmasstransfer.2019.104389
37. *Torkhovskaya T.I., Kostryukova L.V., Tereshkina Y.A., Tikhonova E.G., Morozevich G.E., Plutinskaya A.D., Lupatov A.Yu., Pankratov A.A.* (2021) Chlorin e6 embedded in phospholipid nanoparticles equipped with specific peptides: Interaction with tumor cells with different aminopeptidase N expression. Biomed. Pharmacother., **134**, 111154. DOI: 10.1016/j.biopha.2020.111154
38. *Wang Y., Zheng Y., Zhang L., Wang Q., Zhang D.* (2013) Stability of nanosuspensions in drug delivery. J. Control. Release, **172**(3), 1126-1141. DOI: 10.1016/j.jconrel.2013.08.006
39. *Gai Y., Jiang Y., Long Y., Sun L., Liu Q., Qin C., Zhang Y., Zeng D., Lan X.* (2020) Evaluation of an integrin αvβ3 and aminopeptidase N dual-receptor targeting tracer for breast cancer imaging. Molecular Pharmaceutics, **17**(1), 349-358. DOI: 10.1021/acs.molpharmaceut.9b01134
40. *Das D., Koirala N., Li X., Khan N., Dong F., Zhang W., Mulay P., Shrikhande G., Puskas J., Drazba J., McLennan G.* (2020) Screening of polymer-based drug delivery vehicles targeting folate receptors in triple-negative breast cancer. J. Vasc. Interv. Radiol., **3**1(11), 1866-1873. DOI: 10.1016/j.jvir.2020.05.010
41. *Yoshida T., Oide N., Sakamoto T., Yotsumoto S., Negishi Y., Tsuchiya S., Aramaki Y.* (2006) Induction of cancer cell-specific apoptosis by folate-labeled cationic liposomes. J. Control. Release, **111**(3), 325-332. DOI: 10.1016/j.jconrel.2005.12.016
42. *Lanza P., Felding-Habermann B., Ruggeri Z.M., Zanetti M., Billetta R.* (1997) Selective interaction of a conformationally-constrained Arg-Gly-Asp (RGD) motif with the integrin receptor alphavbeta3 expressed on human tumor cells. Blood Cells Mol. Dis., **23**(2), 230-241. DOI: 10.1006/bcmd.1997.0140
43. *Akhtar K., Broekelmann T.J., Song H., Turk J., Brett T.J., Mecham R.P., Adair-Kirk T.L.* (2011) Oxidative modifications of the C-terminal domain of tropoelastin prevent cell binding. J. Biol. Chem., **286**(15), 13574-13582. DOI: 10.1074/jbc.M110.192088

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ДВУХВЕКТОРНАЯ ТРАНСПОРТНАЯ ФОСФОЛИПИДНАЯ НАНОСИСТЕМА ДОКСОРУБИЦИНА: НАКОПЛЕНИЕ В КЛЕТКАХ РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ *IN VITRO*

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Для лечения рака молочной железы используют различные химиотерапевтические агенты, в том числе антрациклиновый антибиотик доксорубицин, обладающий наряду с цитостатическим действием серьёзными побочными эффектами. Для снижения его негативного влияния на здоровые органы и ткани, повышения его накопления в опухоли доксорубицин был встроен в фосфолипидные наночастицы. Дополнительное использование векторных молекул для направленной доставки к конкретным мишеням может повысить эффективность препарата за счёт более высокого накопления активного вещества в опухолевой ткани. В качестве мишеней таких векторных молекул в данной работе были рассмотрены интегрин $\alpha_v\beta_3$, играющий важную роль в ангиогенезе рака, и рецептор фолиевой кислоты, отвечающий за клеточную дифференцировку и пролиферацию. Была получена фосфолипидная композиция доксорубицина с двумя векторными лигандами — cRGD-пептидом и фолиевой кислотой (NPh-Dox-cRGD-Fol(3,4)). Исследование физических свойств разработанной композиции NPh-Dox-cRGD-Fol(3,4) показало, что средний размер частиц составлял 39,62±4,61 нм, значение ζ-потенциала — 4,17±0,83 мВ; при этом практически весь доксорубицин был встроен в фосфолипидные наночастицы (99,85±0,21%). Одновременное использование двух векторов в композиции приводило к увеличению значения накопления доксорубицина в клетках рака молочной железы MDA-MB-231 практически на 20% по сравнению с композициями, содержащими каждый вектор отдельно (фолиевую кислоту и cRGD-пептид). При этом степень интернализации доксорубицина была на 22% и 24% выше, чем при использовании только фолиевой кислоты и cRGD-пептида соответственно. Цитотоксическое действие на клетки MDA-MB-231 было выше при инкубации с композициями, содержащими фолиевую кислоту в качестве одного вектора (NPh-Dox-Fol(3,4)) и совместно с пептидным (NPh-Dox-cRGD-Fol(3,4)). На диплоидной клеточной линии фибробластов Wi-38 была отмечена значительно меньшая степень цитотоксического действия фосфолипидной композиции, независимо от наличия в ней векторных молекул, по сравнению с субстанцией доксорубицина. Полученные результаты свидетельствуют о перспективности использования двух векторов в одной фосфолипидной композиции для направленной доставки доксорубицина.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: рак молочной железы; фосфолипидные наночастицы; cRGD; интегрин $\alpha_v\beta_3$; химиотерапия; доксорубицин; фолиевый рецептор

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