Experimental ONCOLOGY

COMPARATIVE STUDY OF DYE-LOADED LIPOSOME ACCUMULATION IN SENSITIVE AND RESISTANT HUMAN BREAST CANCER CELLS

S.L. Yefimova[1], I.Yu. Kurilchenko[2], T.N. Tkacheva[1], V.A. Rozhkov[1], A.V. Sorokin[1],*
N.Yu. Lukianova[3], N.A. Bezdenezhnykh[3], Yu.V. Malyukin[1], V.F. Chekhun[3]
[1]Institute for Scintillation Materials, NAS of Ukraine, Kharkov 61001, Ukraine
[2]Slavyansk State Pedagogical University, Slavyansk 84116, Ukraine
[3]R.E. Kavetsky Institute of experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine

The *aim* of this research is to study the dynamics and efficiency of liposome accumulation in sensitive and resistant human breast cancer cells. *Methods*: Methods of fluorescence microscopy, fluorescence microspectroscopy and MTT-test have been used. *Results*: The liposome-to-cell interaction and dye cellular uptake in sensitive, cisplatin-resistant and doxorubicin-resistant MCF-7 human breast cancer cells have been analyzed using time changes in both fluorescence resonance energy transfer signal from the donor probe DiO to the acceptor one DiI preloaded in liposomes and cell image brightness. *Conclusion*: Obtained results show that resistant cells accumulate dye-loaded liposomes more effectively and reveal more effective dye molecule cellular uptake.
Key Words: liposomes, fluorescent probes, human breast cancer cells, fluorescence resonance energy transfer.

Today, the drug targeted delivering is the main task in the effective treatment of many diseases such as cancer, pain syndrome, infectious diseases and others. Drug targeting using suitable carries allows a number of problems associated with drug toxicity and undesirable actions on normal organs and tissues, poor water solubility of many pharmacologically effective compounds and their degradability before therapeutic action to be solved [1–5]. In up-to-date pharmacology and medicine such carriers as polymeric micelles, liposomes, niosomes, microspheres, serum proteins, immunoglobulins, etc. are used [1–5]. Among these carriers, liposomes composed of natural lipids show great potential of effective delivery of drugs and other compounds into the site of action in living body [2, 3]. Liposomes are biodegradable, biologically inert, weakly immunogenic and possess limited intrinsic toxicity [2, 3]. Moreover, liposomes afford a unique opportunity to deliver the drugs into cells by fusion or endocytosis mechanism, i.e. by the atraumatic for cells way [2, 3]. However, despite the evident advantages of drug liposomal form, there are still no in-depth knowledge about preferred interaction of different cell lines with liposomes, rate and efficiency of liposome internalization by cells of different types. Such knowledge is very important in biological researches, pharmacokinetics, clinical medicine for choosing the effective disease treatment and development of new liposomal and other forms of drugs. Fluorescence-based methods, which imply fluorescent probe application, is a powerful tool to visualize

and trace the interaction between carriers and living cells *in vivo* and *in vitro* experiments [6, 7].

In the present paper, the interaction between liposomal vesicles supplied with specific "signal system" based on two fluorescent probes and two types of human breast cancer cells (sensitive and resistant to doxorubicin and cisplatin commonly used to treat some leukemia and cancers of the bladder, breast, stomach, lung and others). To study the dynamics and efficiency of the interaction, we analyze the Electronic Excitation Energy Transfer (EEET) between the fluorescent probes preloaded in liposomes [6, 7]. EEET is a transfer of electronic excitation energy from one molecule (donor) to other molecule (acceptor) without intermediate photon emission through long-range dipole-dipole interactions [6, 7]. Since EEET is dependent on the inverse sixth power of the intermolecular separation [6, 7], it is widely used to study a variety of biological processes associated with the intermolecular distance changing (protein folding, association/dissociation of macromolecules, detection of nuclei acid hybridization, etc.). In present study as a fluorescent probes we use hydrophobic dyes 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) as energy donor and acceptor, respectively (Fig. 1).

The dyes were pre-loaded in lipid bilayers of phosphatidylcholine (PC) liposomes that ensures the required distance between the donor DiO and the acceptor DiI to realize effective EEET [6, 7]. The release of the dyes from liposomes as a result of liposome-to-cell coupling causes a loss of the EEET effect due to the increase of the donor-acceptor distance that is used as a "signal system" to monitor the liposome-to-cell interaction. In our previous paper we reported the results of testing the "signal system" in study that explored the liposome cellular uptake by rat hepato-

cytes, which revealed its high efficiency in monitoring the liposome-to-cell interaction in real time [8].
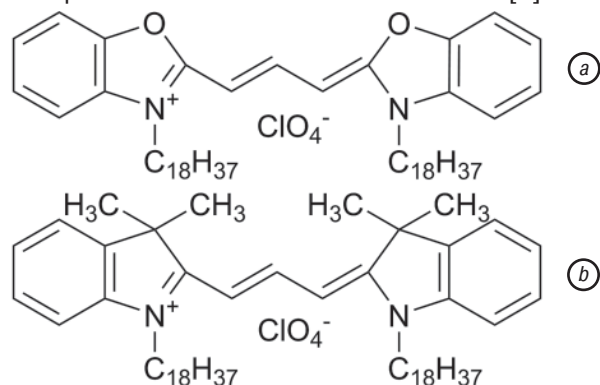

Fig. 1. Molecular structures of (*a*) 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO); (*b*) 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)

MATERIALS AND METHODS

Chemicals. Fluorescent probe DiO was synthesized in the Institute for Scintillation Materials, NAS of Ukraine by Dr. Igor Borovoy. The purity of the dye was controlled by thin layer chromatography. Dye DiI and L-α-Phosphatidylcholine from egg yolk were purchased from Sigma-Aldrich and used without purification. Chloroform (Sigma-Aldrich) used to prepare lipid and dye stock solutions was a spectroscopic grade product.

Preparation of lipid vesicles with DiO and DiI probes. Unilamellar PC lipid vesicles containing DiO and DiI dyes were prepared by the extrusion method [9]. Briefly, appropriate amount of PC (40 mg/ml) and dyes (10^{-3} M) stock solutions in chloroform were mixed in a flask and dried until complete chloroform evaporation. The thin lipid-dyes film was then hydrated with 2 ml of Eagle's medium + 10% fetal calf serum (pH 7.4). Final concentration of PC was 1×10^{-3} M. The obtained lipid-dye suspension was finally extruded through 100 nm pore size polycarbonate filter using a mini-extruder (Avanti Polar Lipids, Inc.). The concentrations of each dye in liposomal suspension were 2×10^{-5} M.

Cell lines and drug treatment. For our studies we used human breast cancer cell line MCF-7 and its sublines resistant to cytotoxic effects of doxorubicin (MCF-7/DOX) and cisplatin (MCF-7/DDP). The cells of the initial MCF-7 line were cultivated in modified Dulbecco's medium ISCOV ("Sigma", Germany) with addition of 10% of fetal calf serum ("Sangva", Ukraine) at the temperature of 37 ˚C and CO_2 concentration of 5%. Cells were reseeded twice a week at the density $2-4 \times 10^4$ cells/cm^2, when cell layer covered about half of the flask surface.

The resistant variants MCF-7/Dox and MCF-7/DDP were originated by growing initial MCF-7 cells with raising concentrations of cisplatin (from 0.01 to 6 μg/ml) or doxorubicin (from 0.1 to 32 μg/ml), respectively. Cisplatin and doxorubicin were added twice a week after reseeding. Every two months, cell survival was analyzed by MTT assay. IC_{50} values for MCF-7 and MCF-7/DDP cells were 0.25 and 1 μg/ml of cisplatin,

respectively, and for MCF-7 and MCF-7/Dox cells — 0.5 and 8 μg/ml of doxorubicin, respectively. Therefore, MCF-7/DDP were 4 times as much resistant to the cytotoxic effect of cisplatin and MCF-7/Dox cells were 16 times as much resistant to the cytotoxic effect doxorubicin as compared with the initial MCF-7 cells.

MTT assay. Sensitivity to antitumor drugs (cisplatin and doxorubicin) was measured every two months using standard MTT-colorimetric test with 3-[4,5,dimethylthiasol-2–1]-2,5-diphenyltetrasolium bromide ("Sigma", Germany) [10].

Cells MCF-7, MCF-7/Dox and MCF-7/DDP were cultured for 24 h and then 5 μL lipid vesicles preloaded with DiO and DiI was added to culture medium. Cells were incubated with dyes-preloaded lipid vesicles for 2.5; 5; 9 and 24 h.

In order to produce cytocentrifuge preparations the drop of cell suspension (500 000 cells/ml) was put on a slide. Glasses were put on special stands and centrifuge at 900 rpm (1 min).

Cell imaging, microspectroscopy and spectroscopy. Cell imaging was performed using inverted fluorescent microscope Olympus IX71 with digital camera Olympus C-5060 at 100x magnification. BP460–490 and BP510–550 filters were used to excite DiO and DiI, respectively. To study EEET in cells incubated with EEET-liposomes, BP460–490 filter was used. Microspectroscopy at the area of interest was carried out using spectral detector USB 4000 (Ocean Optics) connected with Olympus IX71. To obtain fluorescence spectra of the dye-loaded liposomes, a drop of a liposomal suspension on a slide covered with a cover-slip was placed under the objective. Fluorescence was excited using BP460–490 and detected using spectral detector USB 4000.

RESULTS AND DISCUSSION

DiO and DiI dyes exhibit high hydrophobicity and, consequently, extremely poor water solubility [11]. In water solutions such dyes form non-fluorescent aggregates [12]. Forced concentration of the dye molecules in liposome lipid bilayers prevents dye aggregation and their absorption and fluorescence are clearly registered. Moreover, the dye concentration in lipid bilayers ensures the required distance between the donor DiO and acceptor DiI to observe EEET between them. Fluorescent spectra of DiO- and DiI-loaded liposomes and liposomes with both dyes (EEET — liposomes) are presented in Fig. 2.

Fig. 2, curve 3 shows that in the solution containing liposomes with both dyes, the band, which corresponds to the donor DiO emission, is not observed, while fluorescence of the acceptor dye DiI is enhanced sufficiently as the result of EEET from DiO to DiI [6, 7]. The EEET efficiency (E) estimated as $E = 1 - I_{DA}/I_D$, where I_{DA} and I_D are the donor fluorescence intensities in the presence and the absence of the acceptor, respectively, is 92% [6, 7].
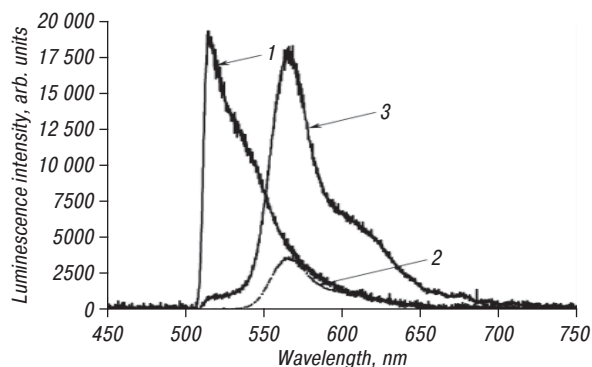
Fig. 2. Fluorescence spectra of liposomes loaded with: 1 — DiO, 2 —DiI, 3 — DiO and DiI (EEET-liposomes). Excitation filter BP 460–490

The damage of liposomes was shown to cause the loss of the EEET effect due to an increase of the donor-acceptor distance [8]. So, we will use the change in a EEET signal as a "signal system" to monitor the dynamics and specificity of liposome — to — cancer cell interactions.

The analyzable parameters are the DiO fluorescence ratio in total fluorescence signal calculated as $I_{DiO}/(I_{DiO} + I_{DiI})$, where I_{DiO} and I_{DiI} are fluorescence intensities of DiO at 510 nm and DiI at 565 nm, respectively, and the change in total brightness of cell fluorescence images that is proportional to the amount of the dye molecules accumulated in cell at different time periods. The total brightness of the cell images obtained at different time periods (2.5; 5; 9 and 24 h) was estimated using image bitmap analysis.

Fluorescence images of cancer cells of each type taken from different time periods of the cell incubation with EEET-liposomes are presented in Fig. 3. As a control point, we present fluorescence images of each cell culture (autofluorescence) before incubation with EEET-liposomes. The change in cell brightness was analyzed with respect to the cell autofluorescence and presented in Fig. 4. Figs. 3 and 4 show that after 24 h incubation, all cell cultures accumulate both fluorescent probes in great amount (bright yellow-green and orange-red fluorescence in Fig. 3 and saturation regions in Fig. 4). However, there are some remarkable differences in dynamics and efficiency of dye cellular uptake by sensitive and resistance cells. Let us analyze the dynamics of liposome-to-cell interaction in sensitive, cisplatin-resistant and doxorubicin-resistant MCF-7 human breast cancer cells.

Sensitive MCF-7 cells. Analysis of the fluorescence images of the cells taken after 2.5 h incubation with EEET-liposomes shows that the liposomes are mainly accumulated on cell membrane (Fig. 3, yellow spots). The fluorescence spectrum recorded from the cells reveals intense DiI fluorescence band as the result of EEET between two dyes in liposomes, while DiO fluorescence band is weakly intense (data not presented). Fig. 5, *a*, represents the diagram of time changes in donor DiO fluorescence ratio and shows that after 2.5 h incubation this value is 0.15 and changes slightly as compared to that in EEET-lipo-

somes (0.1, zero point). Total brightness of the cell image estimated for 2.5 h incubation does not reveal considerable changes (Fig. 4, curve 1). All that points to the fact that the dye release from the liposomes are not observed yet. The increase in the liposome-to-cell incubation time causes the gradual increase in both DiO fluorescence ratio (Fig. 5, *a*) and cell image brightness (Fig. 4, curve 1). After 9 h incubation of MCF-7 cells with EEET-liposomes, the ratio of DiO fluorescence increases up to 0.4 that points to the donor-acceptor distance increase owing to the dye release from the liposomes [6–8]. At the same time, the DiI fluorescence ratio in total fluorescence signal $I_{DiI}/(I_{DiO} + I_{DiI})$ is 0.6. Fig. 2 shows that the DiI direct excitation is negligible as compared to the EEET signal. So, high value of the acceptor DiI fluorescence in total fluorescent signal can be explain by the EEET signal that registered from EEET-liposomes observed in great amount on the surface of cell membranes even after 9h incubation period (yellow-orange spots in Fig. 3).

Two observed experimental facts should be also noted and discussed. First, further increase in the incubation time period does not lead to the total cell brightness increasing (Fig. 4, curve 1) that indicates the saturation of MCF-7 cells with dye-loaded liposomes. We can estimate the saturation time for MCF-7 cells of about 7 h as a cross point of the two straight lines (see Fig. 4). However, the long-time incubation of MCF-7 cells with EEET-liposomes leads to the decrease in DiO fluorescence ratio down to 0.1 (Fig. 5, *a*), meanwhile both DiO and DiI are observed in great amount on the cell membrane surface (Fig. 3).

Liposomes and other drug-delivering carriers can penetrate into cells by such mechanisms as: (1) fusion of vesicle membrane and cell membrane; (2) endocytosis mechanism; (3) adsorption on a cell membrane with a subsequent facilitated diffusion of the active component into the cell; (4) active transport [13]. Chen and co-workers [14] studied the interaction between tumor cells and polymeric micelles loaded with hydrophobic fluorescent probes. They reported the membrane-mediated pathway of hydrophobic probe cellular uptake, implying dyes accumulation in a cell membrane before internalization [14]. We support the same pathway of dye cellular uptake by MCF-7 cells that explains the decrease of DiO fluorescence ratio after 24 h incubation due to EEET observed between DiO and DiI molecules released from liposomes and accumulated in a great amount on the cell membrane surface. Similar effect was observed by Chen et al. [14] after long-time KB cell incubation with dye-preloaded polymeric micelles. At the same time, we did not observe EEET recovering during a long-time incubation of rat hepatocytes with EEET-liposomes [8] that can points to the different efficiency of dye internalization by cells of different types.

Resistant MCF-7/Dox cells. As show Figs. 3, 4 and 5, *b*, the dynamics of the dye-loaded liposome accumulation in this cells sufficiently differs from that of sensitive MCF-7 cells. After 2.5 h incubation, the total brightness of the cells sharply increases and
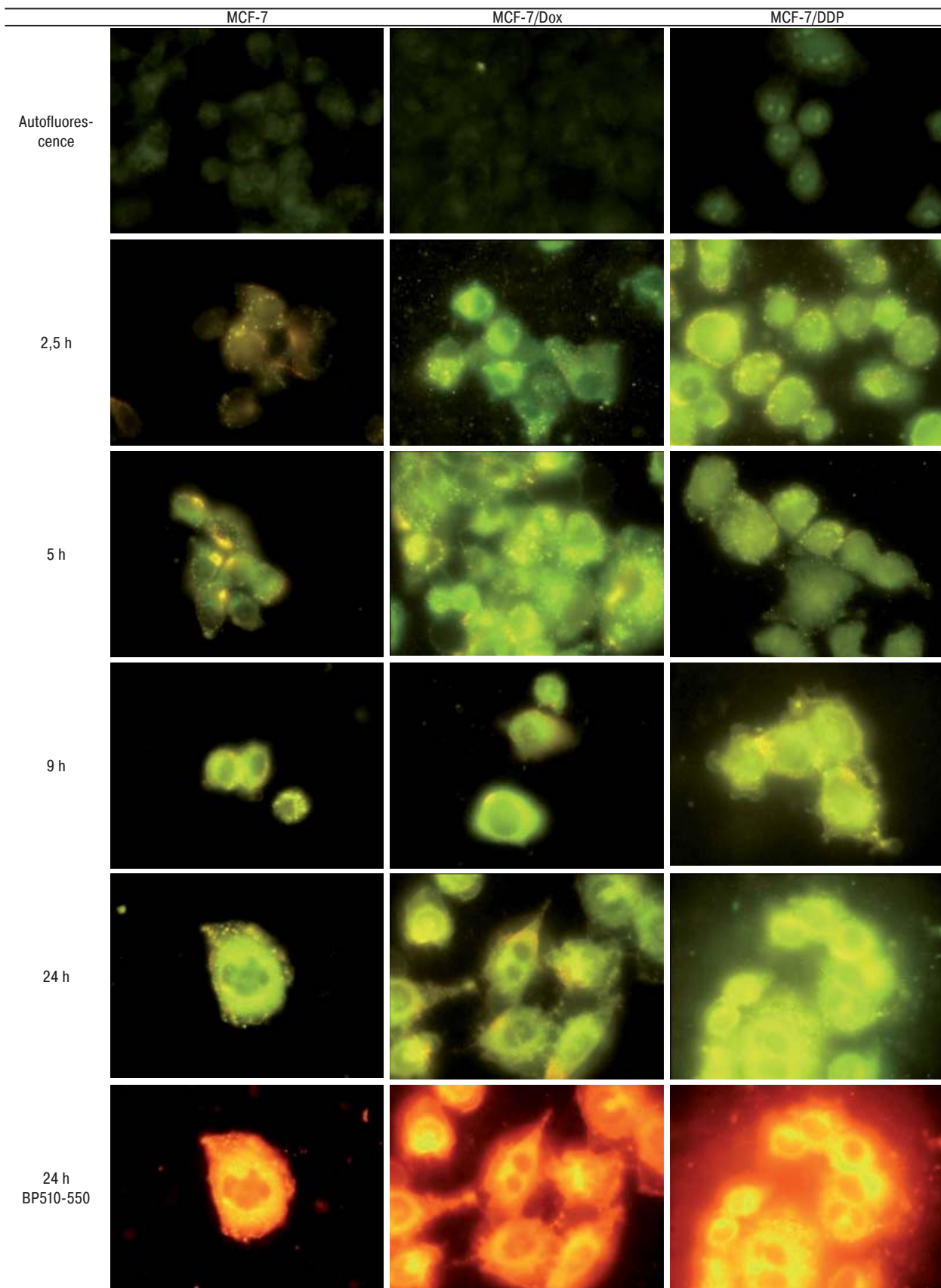
Fig. 3. Fluorescent images (x100) MCF-7, MCF-7/Dox and MCF-7/DDP cells incubated with EEET-liposomes during different time periods. Excitation filter BP 460–490

is 14 times higher than that of sensitive MCF-7 cells at the same incubation time period (Fig. 4, curve 2). The DiO fluorescence ratio is also sharply increases upto 0.55 implying the liposome-to-cell binding and dyes release. Further increase in the incubation time causes the gradual rise of cell image brightness, whereas the DiO fluorescence ratio does not changes remarkably (Figs. 4 and 5, *b*).
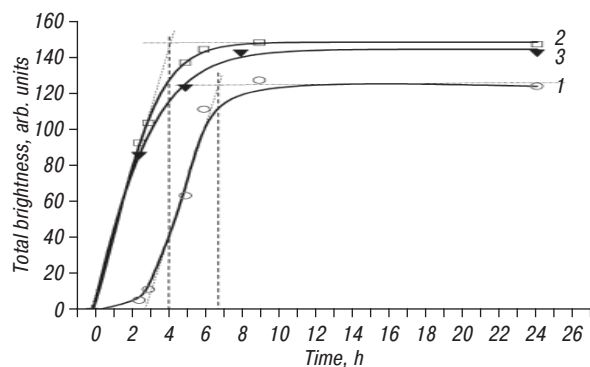
Fig. 4. Total brightness of cell images as a function of cell-to-liposomes incubation time: 1 — MCF-7 cells, 2 — MCF-7/Dox cells, 3 — MCF-7/DDP cells

The saturation time for resistant MCF-7/Dox cells is 4 hours and is 2.5 times faster as compared to the sensitive cells. At the same time, the total amount of dye molecules accumulated in cells, which correlates with the cell brightness, is greater (Fig. 4, curve 2). After long-time incubation (9 and 24 h) the cell brightness does not change, while the DiO fluorescence ratio is slightly decrease down to 0.4 that we associate with EEET recovering owing to dye release from the liposomes and accumulation in a great amount on the membrane surface. It is notable that this EEET recovering is smaller as compared to the sensitive MCF-7 cells that can be explained by the intracellular uptake of the dye molecules that is more effective in resistance MCF-7/Dox cells.

Resistant MCF-7/DDP cells. The dynamics of the dye-loaded liposome accumulation in this cells is similar to that for MCF-7/Dox cells. In 2.5 h incubation both the cell total brightness and DiO fluorescence ratio are sharply increased implying the liposome-to-cell binding and dyes accumulation in cell membrane (Figs. 4, curve 3 and 5, c). Further increase in the incubation time does not cause remarkable changes in the DiO fluorescence signal (Fig. 5, c). The saturation time for resistant MCF-7/DDP cell culture is also about 4 h. After 24 h incubation the total amount of the dye molecules accumulated in cells is also higher as compared to the sensitive MCF-7 cells (Fig. 4, curve 3). At the same time, the EEET recovering is not observed that suggests the effective dye molecules intracellular uptake.

In conclusion, the time changes in EEET signal between two fluorescent probes preloaded in PC liposomes and cell image total brightness were used to visualize and study the dynamics of liposome accumulation in sensitive, cisplatin-resistant and doxorubicin-resistant MCF-7 human breast cancer cells. Our results show that there are sufficient differences in dynamics and efficiency of the liposome-to-cell interaction and dye molecule internalization in sensitive and resistant cells. In case of resistant MCF-7/Dox and MCF-7/DDP cells, the liposome-to-cell interaction is more effective: the time of cell saturation with liposomes is shorter, whereas the amount of liposomes and, consequently, dye molecules accumulated on a cell membrane that correlates with the

total cell brightness is higher. The rate of dye release from the liposomes is higher. At the same time, the dye molecules intracellular uptake appears to be more effective.
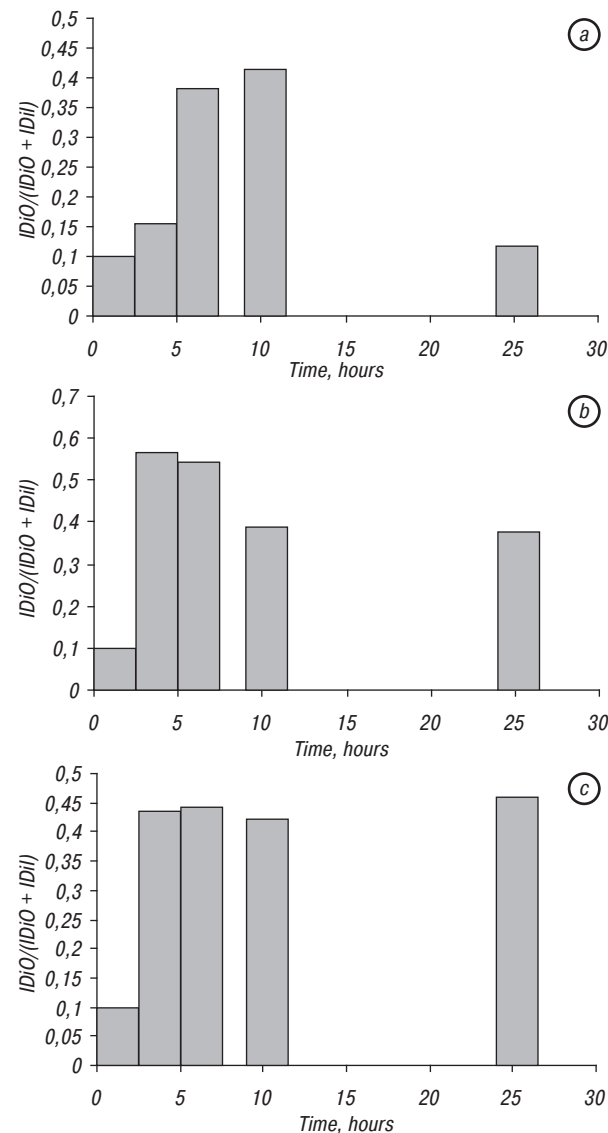

Fig. 5. Time chances in DiO fluorescence ratio $I_{DiO}/(I_{DiO} + I_{DiI})$ for MCF-7 cells (*a*), MCF-7/Dox cells (*b*) and MCF-7/DDP cells (*c*)

REFERENCES

1. You C-C, Chompoosor A, Rotello VM. The biomacromolecule — nanoparticle interface. NanoToday 2007; **2**: 34–43.

2. Kozubek A, Gubernator J, Przeworska E, Stasiuk M. Liposomal drug delivery, a novel approach: PLARosomes. Acta Biochimica Polonica 2000; **47**: 639–49.

3. Goyal P, Goyal K, Kumar SGV, *et al.* Liposomal drug delivery systems — clinical applications. Acta Pharm 2005; **55**: 1–25.

4. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discovery 2010; **9**: 615–27.

5. Nishiyama N, Kataoka K. Current state, achievements and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther 2006; **112**: 630–48.

6. Lakowicz JR. Principles of Fluorescence Spectroscopy. New York, Boston, Dordrecht, London, Moscow: Kluwer Academic/Plenum Publishers, 1999. 960 p.

7. Valeur B. Molecular Fluorescence. Principles and Applications. Weinheim: Wiley, 2002. 468 p.

8. Yefimova SL, Lebed AS, Guralchuk GYa, *et al.* Nanoscale liposomal container with a "signal system" for substances delivering in living cells. Biopolymers Cell 2011; **27**: 47–52.

9. Mui B, Chow L, Hope MJ. Extrusion technique to generate liposomes of defined size. Meth Enzymol 2003; **367**: 3–14.

10. Ni J, Hollander D. Application of the MTT-assay to functional studies of mouse intestinal intraepithelial lymphocytes. J Clin Lab Anal 1996; **10**: 42–52.

11. Hauglang RP. Handbook of fluorescent probes and research products. New York: Molecular probes, 2002. 920 p.

12. Lebed' AS, Yefimova SL, Guralchuk GYa, *et al.* Effect of hydrophocicity of cationic carbocyanine dyes DiOCn on their binding to anionic surfactant micelles. J Appl Spectroscopy 2010; **77**: 183–8.

13. Kaplun AP, Le Bang S, Krasnopolsky YuM, Shvets VI. Liposomes and other nanoparticles as the drug delivery systems. Voprosy Med Khimiyi 1999; **1**: 3–12 (in Russian)

14. Chen H, Kim S, Li L, *et al.* Release of hydrophobic molecules from polymer micelles into cell membranes revealed by Föster resonance energy transfer imaging. PNAS 2008; **105**: 6596–601.