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DISTRIBUTION AND ACCUMULATION OF LIPOSOMAL FORM OF DOXORUBICIN IN BREAST CANCER CELLS OF MCF-7 LINE

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***Aim*: To study distribution and accumulation of liposomal form of doxorubicin in human breast cancer cells of MCF-7 line and Dox-resistant subline MCF-7/Dox. *Methods*: High performance liquid chromatography and laser confocal microscopy were used. *Results*: It has been shown that conventional form of doxorubicin was more efficiently delivered to the MCF-7 cells already after 30 min of incubation amounting to its maximum concentration after 4 h. MCF-7/Dox cells are characterized by lower doxorubicin accumulation rate compared with parental cells. The quantity of accumulated liposomal form of doxorubicin is high in MCF-7 cells, and, what is important, Dox-resistant cells accumulated higher levels of liposomal form of doxorubicin than its conventional form. *Conclusion*: It has been shown that intracellular distribution and accumulation of liposomal forms of doxorubicin in parental and Dox-resistant MCF-7 cells differs from that of conventional doxorubicin.**
***Key Words*: drug resistance, human breast cancer, MCF-7 cells, LipDOX.**

Chemotherapy remains the standard treatment of both hematopoietic and solid tumors. Many cancers are characterized by an initial sensitivity to chemotherapy; however, acquired resistance to therapy invariably leads to patient relapse though the expansion of a multidrug-resistant population of cancer cells. The development of multiple drug resistance (MDR) remains the main problem in treatment of cancer patients [1]. Drug resistance develops toward practically each effective anticancer drug (ACD) with an involvement of numerous mechanisms: reduced drug accumulation; alterations in drug target; increased repair of drug-induced damage and inhibition of apoptotic signaling pathways [2–4].

An efficacy of anticancer therapy may be achieved by many ways, among which one could mention the generation of new forms of ACD, in particular liposomal ones. Preclinical studies have shown pharmacological benefits of liposomal forms of anthracycline, namely: pharmacokinetic patterns that are analogous to these for prolonged infusion of conventional anthracyclines, larger accumulation of the drug in tumor tissue and lower general toxicity compared to the conventional forms. The results of clinical trials have also shown more expressed cytotoxic action of liposomal forms of drug on cancer cells and decreased general toxicity of patient's organism. It is very important, that drug resistance may be partially reversed upon the use of liposomal forms [5–7]. Liposomal systems for drug delivery may overcome an activity of transport MDR proteins even in highly resistant tumors. Liposomes deliver the drug to the cytoplasm of the cancer cells by endocytosis, thus interaction with Pgp which is expressed on plasma membrane, is significantly decreased. Michieli *et al.* [8] have observed that treatment of cell lines that overexpressed Pgp, by liposomal daunorubicin caused higher intracellular accumulation of the drug compared to the conventional form what led to 4–5 fold elevated cytotoxic effect. The molecular mechanisms of the binding and internalization of liposomal drugs are not fully understood. However, it is generally accepted that the majority of liposomes enter cells through endocytotic pathway. The mechanism of this effect remains incompletely studied yet. It has been shown that the colon cancer cells and their resistant analogs do not differ by DNA damage degree upon the action of conventional form of doxorubicin (Dox) and its liposomal form (LipDox) [9]. The authors supposed that enhancement of the LipDox biological activity could be explained by existence of its other targets, not related to DNA. Experimental results suggest that the enhancement of sensitivity of MDR cells using LipDox may be explained by an increase in cell drug incorporation and by an intracellular drug redistribution [10–11]. Decreased accumulation of ACD and their increased efflux from the cell is a characteristic pattern of resistant cells even at low degree of the resistance [12]. Intracellular accumulation of ACDs is very important for evaluation of their cytotoxic action. The studies of ACD accumulation, retention, distribution in the cell, and efflux from the cell is also important because such data may be useful for the development of therapeutic schemes.

MATERIALS AND METHODS

The study was carried out on human breast cancer cells of MCF-7 line and its resistant subline MCF-7/Dox. The resistant subline was produced by exposing MCF-7 cells in the presence of gradually increasing concentrations of Dox ("Ebeve", Austria) at the range from 0.1 to 32 mg/ml [13]. The MCF-7/Dox cells were 8-fold more resistant to Dox than the parental cells.

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Abbreviatons used: ACD – anticancer drug; Dox – conventional forms of doxorubicin; DoxLip – liposomal forms of doxorubicin; MDR – multiple drug resistance; P-gp – P-glycoprotein.

Quantitative evaluation of LipDox and Dox accumulation by MCF-7 and MCF-7/Dox cells has been performed by the method of high performance liquid chromatography (HPLC). The cells were cultured for 30 min, 1, 4 h in ISCOW culture medium with mentioned preparations at IC30 concentrations (that was equal to 2 and 4 μg/ml for parental and resistant cell lines, respectively). Then the cells were washed twice and were incubated for 24 h at Dox-free culture medium.

Then incubation culture medium was collected, filtered through 0.2 μm filter and analyzed for LipDox and Dox content with the use of liquid chromatograph Agilent Technology 1200 (Germany) with diode array detector at wave length of 235 nm and fluorometric detector. Excitation wavelength (Ex=460), emission wavelength (Em= 550). Column Zorbax SB-C8 Stable Bond Analytical 4, 6 x 250 mm 5-Micron was used. Flow rate of eluent (acetonitrile and buffer solution with pH 2.05) was 1 ml/min, column temperature was 30 °C, and gradient regimen for 15 min was applied. At least 5 chromatograms per each sample were obtained. Calculation of the concentrations were performed by the method of absolute standard — by area of pike for standard Dox solution prepared with the use of Dox substance. As the control, culture medium of cells grown without Dox addition or Dox/acetonitril solution were used.

Determination of LipDox and Dox intracellular distribution was carried out by laser confocal microscopy. The cells were seeded on coverslips overnight and then cultured with Dox or LipDox as described above, washed in physiologic solution and fixed in 4% paraformaldehyde solution for 20 min at 4 °C. Next, the cells were washed and placed in Faramount Aqueous Mounting Medium (DakoCytomation, Denmark). The preparations were examined with the use of laser confocal microscope LSM 510 META (CARL ZEISS, Germany) at excitation wave length of 540 nm and emission wave length of 590 nm.

RESULTS AND DISCUSSION

Quantitative evaluation of LipDox and Dox accumulation by the cells of MCF-7 and MCF-7/Dox lines. With the use of HPLC we have performed the study of LipDox and Dox accumulation in parental MCF-7 cells and in resistant MCF-7/Dox subline. It has been shown that after 30 min incubation of the cells with LipDox or Dox the latest is accumulated in MCF-7/Dox cells in 3.7 times higher than Dox (37% *versus* 10%), while MCF-7 cells accumulated larger amounts of Dox compared with its liposomal form (Table 1).

Table 1. Kinetics of Dox and LipDox accumulation (%) in MCF-7 and MCF-7/Dox cells

Cell line	Incubation period					
	30 min		1 h		4 h	
	Dox	LipDox	Dox	LipDox	Dox	LipDox
MCF-7	46.66 ± 0.33	34 ± 0.57	55.66 ± 0.88	70 ± 1.15	64.66 ± 0.88	82 ± 1.73
MCF-7/Dox	10.66 ± 0.66	36.66 ± 0.66	34.66 ± 0.33	44 ± 1.15	27.6 ± 1.45	59.33 ± 0.66

After 1 h incubation with the drugs, in the cells of parental line LipDox accumulation was higher than that of Dox (70% *versus* 56%). In resistant cells the level of LipDox accumulation was unaltered (45%), while Dox concentration elevated up to 35%. Accumulation levels of Dox and LipDox at this time period could be related to different ways of cell penetration: by direct diffusion (mostly characteristic for Dox), or by endocytosis for liposomal form [14–17].

After 4 h of incubation in both cell lines maximal accumulation of the drugs has been registered: for LipDox in MCF-7/Dox subline up to 60%, and in MCF-7 cells — up to 83%, while for Dox these values yielded 28% and 65% for parental and resistant cells respectively; this fact pointed at increase efflux of the drug from the cells with resistant phenotype (Fig. 1).

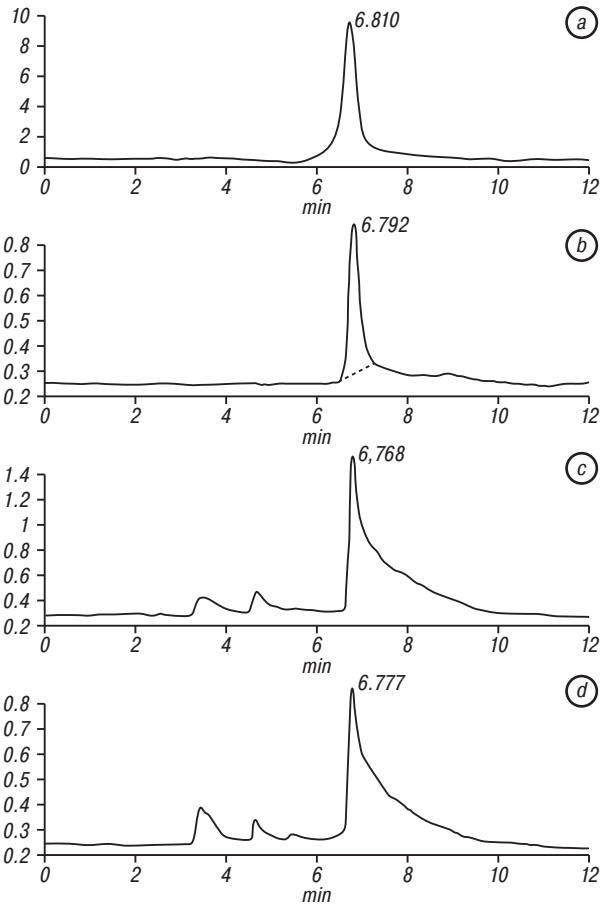

Fig. 1. Chromatographic analysis of doxorubicin content in cell culture medium during 4 h: a) MCF-7/Dox cells treated with Dox; b) MCF-7 cells treated with Dox; c) MCF-7/Dox cells treated with LipDox; d) MCF-7 cells treated with LipDox

Maximal accumulation of the drugs in 4 h incubation period has been supported by determination of their concentration after 5 h of the cells incubation with LipDox and Dox. It have been shown the decrease of conventional forms of the drugs in parental and resistant cells; however, LipDox content remained high enough even in MCF-7/Dox cells.

At 4-h long incubation of the cells with the drugs and the following 24-h incubation with Dox-free culture medium, the Dox content in the cells sharply decreased and could not be detected by HPLC contrary to LipDox which efflux from cells occurred more slowly.

So, the study has revealed that Dox accumulates in MCF-7 cells at large amounts just in 30 min of in-

cubation, and its concentration in the cells reaches its maximum in 4 h. The resistant cells accumulated much lower amounts of Dox. In regard to LipDox, its quantity is high enough in sensitive cells, and is much higher in resistant MCF-7/Dox cells compared to conventional form of the drug.

Determination of LipDox and Dox intracellular distribution in MCF-7 and MCF-7/Dox cells. Intracellular drug accumulation is a complex process including drug uptake into the cell, retention and distribution in the cell, and efflux from the cell [18, 19]. Intracellular accumulation of ACDs is an important parameter for evaluation of their cytotoxic action. The use of laser confocal microscopy for determination of intracellular distribution of ACDs is beneficial compared to fluorescent microscopy due to higher resolution capacity and elimination of epifluorescence. It is known that doxorubicin is able to self fluorescence, what allows determining its distribution in cells without additional stainers. Confocal cell images were used to determine intracellular LipDox and Dox localization and accumulation in MCF-7 and MCF-7/Dox sublines after incubation with drugs for 30 min, 1 h, 4 h, and then incubated in Dox-free medium for additional 24 h.

There are clearly determined three types of APC distribution: nuclear, cytoplasmic and mixed. The first one was presented by fluorescence localized in nucleus, having its shape and size. The second type looks like diffuse fluorescence of cytoplasm, without fluorescence in nuclei. The mixed type was mostly characteristic for the cells studied by us at certain time periods, and fluorescence was detected both in cell nucleus and cytoplasm.

Already after 30 min incubation of parental MCF-7 cells with Dox we have registered Dox fluorescence predominantly in nuclei, with homogenous type of fluorescence in all area (Fig. 2, a). At the same time in resistant MCF-7/Dox cells fluorescence was observed in the cytoplasm with higher fluorescence intensity in the perinuclear region (Fig. 2, e).

After 1 h of incubation Dox accumulated in the nuclei of large majority of the MCF-7 cells, fluorescence became more intense, especially at periphery of the nucleus (Fig. 2, b). Fluorescence of Dox in MCF-7/Dox cells during 1 h incubation period was mostly observed in the perinuclear region of cytoplasm and in the nuclei (Fig. 2, f). Fluorescence intensity increased in first 30 min and remained stable for 1 h.

The study has shown that after 4 h incubation with Dox fluorescence still had nuclear localization but its intensity decreased, and one could observe single inclusions with intense fluorescence in the cell cytoplasm located near plasma membrane (Fig. 2, c), possibly, exosomes for removal of drug metabolites from the cells. In resistant MCF-7/Dox cells intense fluorescence of the drugs was observed in perinuclear region as well as diffuse or point-like patterns in cytoplasm and in the nuclei (Fig. 2, g). MCF-7/Dox cells differed from parental MCF-7 cells by the presence of significantly lower level of fluorescence intensity.

Cultivation of the cells with the drugs for 4 h and with the next incubation in doxorubicin-free medium has demonstrated that after 24 h weak Dox fluorescence could be detected only in single cells of parental MCF-7 cells, but none — in resistant cell line (Fig. 2, d; Fig. 2, h).

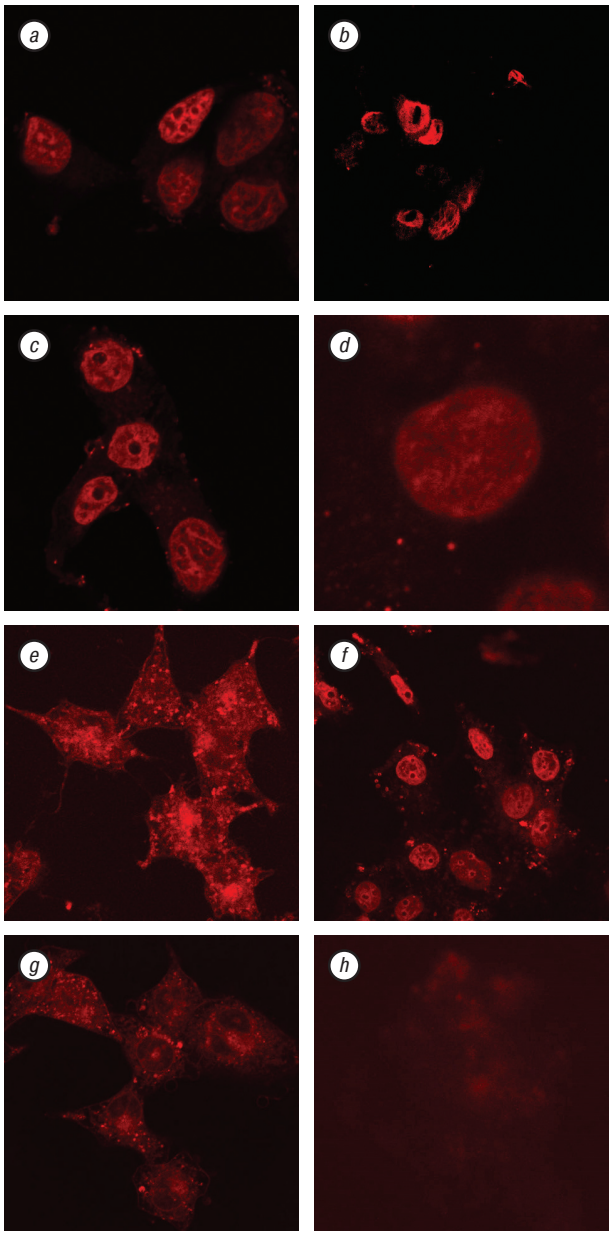

Fig. 2. *In vitro* confocal images of subcellular Dox fluorescence distribution in parental MCF-7 cells (a–d) and resistance MCF-7/Dox cells (e–h): a–d — Dox fluorescence predominantly in nuclei; e–h — Dox fluorescence observed in perinuclear region of cytoplasm, in nuclei and as separate cytoplasmic vesicles

So, one may conclude that Dox fluorescence is registered predominantly in the nuclei of MCF-7 cells, while in resistant cells Dox is mostly accumulated in cell cytoplasm, with the highest intensity in perinuclear region or in point-like cytoplasmic inclusion. Fluorescence in the nuclei is registered only after 1 h of incubation. Lower Dox fluorescence is caused by functioning of the system for reverse ACD transport from resistant cells due to Pgp expression.

Intracellular distribution of ACD is modified in a large number of drug resistant cell lines [18, 19]. Predominantly diffuse localization of Dox in the cyto-

plasm or perinuclear region of the resistant cells could be explained, firstly, by significant Pgp expression not only on plasma membrane, but also on nuclear membrane [20]. Secondly, recent studies suggests that Pgp has expressed also on membranes of Golgi apparatus, where Pgp promotes ACD accumulation inside this organelle with its following removal from the vesicles separated from Golgi complex [21]. The location of well developed 2–3 loci Golgi complex in perinuclear space of resistant cells demonstrated by us using electron microscopy, supports the possibility of Dox accumulation in structural elements of this organelle [22].

When MCF-7 cells were treated with LipDox there have been detected the changes of patterns of fluorescence (Fig. 3, a–d; Fig. 3, e–h). After 30 min treatment LipDox is distributed by all cytoplasm area of MCF-7 and MCF-7/Dox cells, along with generation of large regions of fluorescence in perinuclear region of cytoplasm of MCF-7 cells (Fig. 3, a), or characteristic diffuse staining of cytoplasm of resistant cells with small point-like inclusions (Fig. 3, e). Local accumulation of LipDox may be related to endocytosis of the drug in the cells in addition to its diffusion. It's necessary to note that fluorescence level of LipDox-treated cells is lower than that of Dox-treated cells and was detected in lower number of the cells, what could point on slower entrance of LipDox in cells compared to Dox.

After 1 h incubation with LipDox the latest could be detected in the cytoplasm and nuclei of low percent of parental cells (Fig. 3, b) or as diffuse fluorescence in cytoplasm of MCF-7/Dox cells (Fig. 3, f). It's necessary to note that in MCF-7/Dox cells the fluorescence degree is increased, and there appeared large fluorescent zones in perinuclear space.

If the cells were cultivated with the drug for 4 h, there has been shown a weak fluorescence in nuclei of MCF-7 cells (Fig. 3, c), while high fluorescence intensity in MCF-7/Dox cells was located in nuclei as discrete regions, or it formed local clusters in cytoplasm (Fig. 3, g). Such patterns of fluorescence may respond to vacuoles and endosomes that were observed at large quantities during electron microscopy study. The presence of such vesicles may serve as an evidence of enhanced membrane traffic in drug resistant cells.

Further cultivation of the cells without the drugs has shown that after 24 h very weak diffuse fluorescence could be detected in parental MCF-7 cells (Fig. 3, d), while in resistant cells fluorescence could be observed not only in cytoplasm, but also in nuclei as diffuse or point-like patterns of fluorescence (Fig. 3, h).

In conclusion, our study has demonstrated that accumulation and intracellular distribution of liposomal forms of doxorubicin differs in parental and Dox-resistant MCF-7 cells from that of its conventional form. It has been shown that liposomal form of drug accumulates at higher degree and in more prolonged fashion in resistant MCF-7/Dox cells compared to conventional Dox.

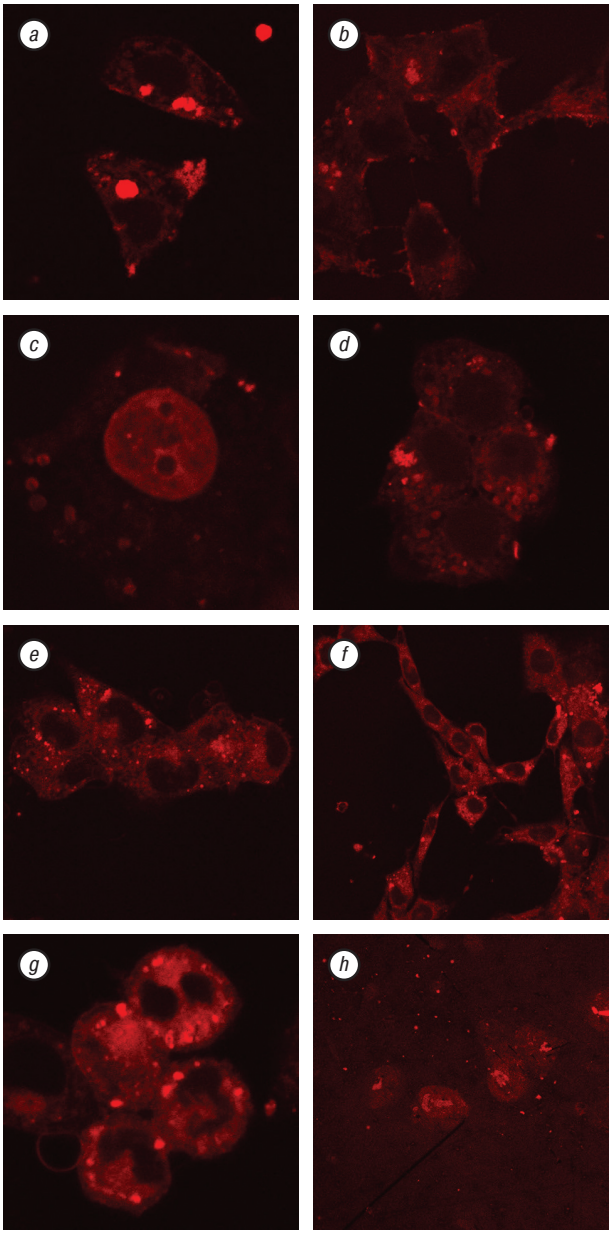

Fig. 3. *In vitro* confocal images of subcellular LipDox fluorescence distribution in parental MCF-7 cells (a–d) and resistance MCF-7/Dox cells (e-h): a–d — Dox fluorescence in cytoplasms and after 4 hour in nuclei (c); e–h — diffuse and point-like fluorescence localization of Dox in cytoplasm and in perinuclear region

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